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Award Number: DAMD17-03-1-0042

TITLE: Molecular Effects of I3C/DIM in Prostate Cancer Cells

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REPORT DATE: April 2005

TYPE OF REPORT: Annual

20060223 083

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 074-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE April 2005	3. REPORT TYPE AND DATES COVERED Annual (1 Apr 2004 - 31 Mar 2005)	
4. TITLE AND SUBTITLE Molecular Effects of I3C/DIM in Prostate Cancer Cells		5. FUNDING NUMBERS DAMD17-03-1-0042	
6. AUTHOR(S) Fazlul Sarkar, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wayne State University Detroit, Michigan 48201		8. PERFORMING ORGANIZATION REPORT NUMBER	
E-Mail: fsarkar@med.wayne.edu			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) <p>Our previous data have shown that I3C inhibits cell growth and induces apoptosis in cancer cells. We have also observed a reduction in the activated Akt and the activity of NF-κB in prostate cancer cells treated with I3C. By gene transfection and kinase assay, we have found that the inhibition of NF-κB by I3C and DIM was partly mediated through the inactivation of Akt. I3C and DIM inhibited phosphorylation of Akt, inactivated NF-κB, and regulated the expression of genes related to cell growth and apoptosis. However, no significant such effects were observed in non-tumorigenic prostate epithelial cells. To further investigate the mechanism(s) by which I3C and DIM inhibit NF-κB, we have transfected IKKβ, IKKα, MEKK, and NIK cDNA into PC-3 prostate cancer cells and tested the alteration in the expression of IKKβ, IκBα, p-IκBα, NF-κB, IKKα, and MEKK in parental and transfected PC-3 cells treated with I3C or DIM. We found that I3C and DIM inhibited the expression of IKKβ, the subsequent phosphorylation of IκBα, and the nuclear localization and activation of NF-κB, resulting in inhibition of cell growth and induction of apoptosis in prostate cancer cells. These results along with our previous findings suggest that I3C and DIM may be potent agents for the prevention and/or treatment of prostate cancer.</p>			
14. SUBJECT TERMS No subject terms provided.		15. NUMBER OF PAGES 47	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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Introduction

Epidemiological and dietary studies have shown an association between high dietary intakes of vegetables and decreased prostate cancer risk (1). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family appears to be most effective at reducing the risk of cancers (2). Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family, and it is readily converted to its dimeric product, 3,3'-diindolylmethane (DIM). I3C and DIM have been found to inhibit the carcinogenesis in animal experiments and the growth of various cancer cells in culture (3, 4). The data from our laboratory and others have shown that I3C induces apoptotic cell death in cancer cells by up-regulation of Bax and p21^{WAF1}, and down-regulation of Bcl-2 and Bcl_{XL} (5, 6). We have found that I3C and DIM regulates the expression of genes, which are critically involved in the control of cell growth, cell cycle, apoptosis, signal transduction, Phase I and II enzymes, and oncogenesis (7). We have also observed a drastic reduction in the activated form of the Akt (6), and DNA-binding activity of NF-κB in prostate cancer cells treated with I3C or DIM (5), suggesting the effects of I3C and DIM on Akt and NF-κB pathways which are two of the important pathways in cancer cells. Based on these results, we hypothesize that I3C and DIM function as inhibitors of NF-κB, which may be due to the inactivation of Akt-related signaling molecules that are important regulators of NF-κB DNA-binding activity, and ultimately lead to the induction of apoptotic processes. To test our hypotheses we will investigate how I3C and DIM inhibit Akt activation leading to apoptotic cell death of prostate cancer cells and how the Akt and NF-κB pathways may cross-talk during the ultimate demise of prostate cancer cells induced by I3C and DIM. We will also investigate the effects of I3C and DIM on the NF-κB upstream signaling molecules such as MEKK, MEK, NIK and IKK, which are known to play important roles in the activation of NF-κB. The results of this study will provide us with not only the information regarding the molecular mechanism(s) of action of I3C and DIM in prostate cancer cells, but will also provide us with molecular markers that may be useful for monitoring effectiveness of I3C and DIM during in vivo animal or human studies. In addition, these results should identify novel pathways, which could be targeted for the development of molecular therapeutic approaches for the prevention and/or treatment of prostate cancer in the future. These results will also provide important molecular evidence, which could be exploited for sensitization of prostate cancer cells to commonly available chemotherapeutic agents.

Body of Report

The original statement of work in the proposal is listed below:

Task-1: We will determine whether treatment of prostate epithelial cells with I3C/DIM elicit responses leading to modulation of NF-κB and investigate the molecular mechanism of NF-κB inactivation as proposed under specific aim-1. This investigation will be conducted using both I3C as well as DIM in androgen sensitive (LNCaP) and androgen independent (PC-3) prostate cancer cells and the data will be compared to those obtained from non-tumorigenic prostate epithelial cells (CRL2221). Task 1 will take 8 months to complete. Time Period 0-8 months.

Task-2: We will determine whether constitutive activation of Akt (by gene transfection studies) increases NF-κB activation in prostate epithelial cells as indicated under task-1, and thereby inhibits apoptotic processes induced by I3C/DIM. We will conduct transient transfection experiments followed by establishing stably transfected cells in the future in order to determine the inhibition of I3C/DIM induced cell death in those cells that over-express Akt and NF-κB. Task 2 will take 6-12 months to complete. Time Period 8-20 months.

Task-3: Once we complete task-1 and 2, we will start working on task 3, which will have significant number of transfection experiments. Task 3 will determine whether treatment of prostate epithelial cells with I3C/DIM will elicit responses that may determine causal association between the inactivation of Akt and NF-κB with the induction of apoptosis. Furthermore, we will investigate what signaling pathways are modified by I3C/DIM that leads to NF-κB inactivation. We will investigate different kinases that are involved in the NF-κB pathway, particularly the status of IKK, MAP kinase-MEK, MEKK1 and NIK. In order to establish cause and effect relationships of these genes with the ultimate effect of I3C/DIM, several transfection experiments are planned which will be very time consuming and labor intensive. Hence this task will take considerably more time. We expect to complete this task within 12 months. Time Period 20-32 months.

Task-4: Task-4 will be focused on to complete all data analysis, manuscript writing and for the development of novel ideas that may be submitted to Federal Agencies for further continued funding in a larger scale encompassing animal and human investigations to test (a) whether I3C/DIM could be an effective agent for the prevention and/or for the treatment of tumors in animal models, (b) whether I3C/DIM pre-exposure of prostate cancer cells will sensitize these cells to commonly available chemotherapeutic agents, and finally (c) whether I3C/DIM could be an effective agent for the prevention and/or treatment of human prostate cancer. Task 4 will be completed within the time period of 32-36 months.

In the last annual report, we have provided the evidences to support the research accomplishments we made last year and indicated that (a) the task-1 has been fully completed; (b) we have completed majority parts of task-2 and plan to complete this task in the second year; (c) we plan to complete task-3 in the second and third year; and (d) once the tasks 1-3 are all

completed, we will make progress on task-4 to complete all experiments, data analysis and manuscript writing during the third year of funding.

We are now reporting the research accomplishments associated with task 2-4 outlined in the Statement of Work.

Task-2. Our task-2 was to investigate the relationship between Akt and NF-κB, the effects of I3C and DIM on Akt and NF-κB pathways, and the effects of I3C and DIM on apoptosis through regulation of Akt and NF-κB pathways.

A. The effects of DIM.

By immunoprecipitation, kinase assay, and Western Blot analysis, we have found that DIM inhibited phosphorylation of Akt, which has been known to be a regulator of NF-κB. To investigate the relationship between Akt and NF-κB, we have conducted transfection experiments with Akt (wild, mutant, or constitutively activated) expression vector and NF-κB-Luc vector that contains NF-κB binding and reporter sequences, and measured the activity of luciferase as well as NF-κB DNA binding activity in transfected prostate cancer cells. We have also detected the effects of DIM on Akt and NF-κB in transfected and un-transfected prostate cancer cells. We found that Akt transfection up-regulated activity of NF-κB as demonstrated by luciferase assay and EMSA. We also found that DIM inhibited the activation of NF-κB induced by Akt transfection, suggesting that the inhibition of NF-κB by DIM is partly mediated by Akt pathway.

We have also investigated the pro-apoptotic effect of DIM in PC-3 prostate cancer cells and Akt transfected PC-3 cells. We found that DIM induced apoptosis in both parental and Akt transfected PC-3 cancer cells. However, Akt transfection showed inhibitory effect on the apoptosis induced by DIM, suggesting that the pro-apoptotic effect of DIM is mediated by Akt and NF-κB pathways. We also investigated the effect of DIM on the expression of genes related to the control of cell growth and apoptosis.

The results of our study described above have been published in the journal "Frontiers in Bioscience" (8; please also see Appendices 1). The summary of our results in the form of an abstract is presented below.

Abstract: Prostate cancer is the second leading cause of cancer related deaths in men in the United States. I3C and its *in vivo* dimeric product, DIM, have been found to inhibit the growth of prostate cancer cells. However, the molecular mechanism(s) by which DIM elicits its effects on prostate cancer cells has not been fully elucidated. We have previously shown that I3C induces apoptosis and inhibits the activation of NF-κB pathway, which could be mediated via Akt signaling pathway. In this study, we investigated whether there is any cross-talk between Akt and NF-κB during DIM-induced apoptosis in PC-3 prostate cancer cells. We found that DIM inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells but not in non-tumorigenic CRL2221 human prostate epithelial cells. DIM also inhibited EGFR expression, PI3K kinase activity, and Akt activation, and abrogated the EGF-induced activation of PI3K in prostate cancer cells. NF-κB DNA-binding analysis and transfection studies with Akt cDNA constructs revealed that Akt transfection resulted in the induction of NF-κB activity and this was inhibited by DIM treatment. DIM treatment also showed significant induction of apoptosis in non-transfected cells compared to Akt and Akt-Myr transfected prostate cancer cells. From these results, we conclude that the inhibition of Akt and NF-κB activity and their cross-talk is a novel

mechanism by which DIM inhibits cell growth and induces apoptotic processes in prostate cancer cells but not in non-tumorigenic prostate epithelial cells.

B. The effects of I3C.

We have also investigated whether I3C have similar effects as DIM on Akt, NF- κ B, and apoptotic pathways. We found the I3C inhibited the growth of PC-3 prostate cancer cells and induced apoptosis in PC-3 cells in dose- and time-dependent manners (Figure 1 and 2).

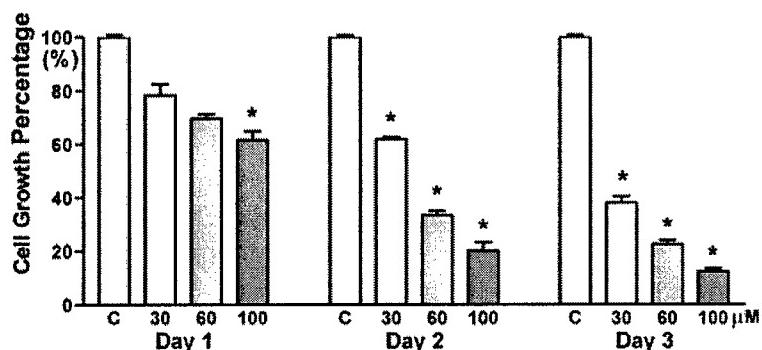


Figure 1: MTT assay showing the significant inhibition of cell growth by I3C in PC-3 cells (C: control; 30, 60, 100: 30, 60, 100 μ M I3C; *: $p<0.05$).

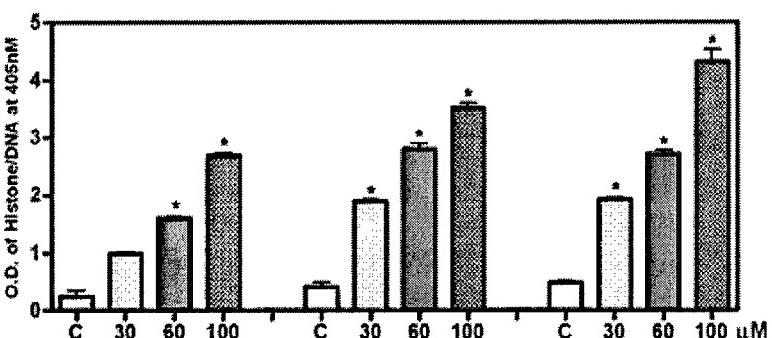


Figure 2: Apoptosis-ELISA assay showed the significant induction of apoptosis by I3C in PC-3 cells (C: control; 30, 60, 100: 30, 60, 100 μ M I3C; *: $p<0.05$).

To investigate the molecular mechanism by which I3C inhibited prostate cancer growth and induced apoptosis, we tested the alteration of Akt and NF- κ B, two important regulators of cell growth and apoptosis, in I3C treated PC-3 prostate cancer cells. We found that I3C inhibited the phosphorylation of Akt (Figure 3) and the DNA binding activity of NF- κ B (Figure 4).

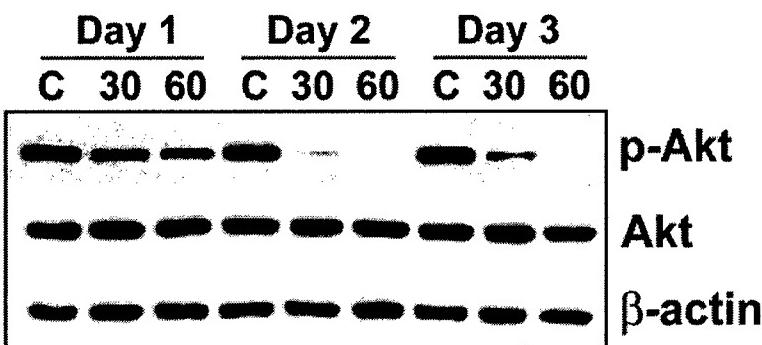


Figure 3: Western Blot analysis showed that I3C treatment for 48-72 hours significantly inhibited the phosphorylation of Akt in PC-3 prostate cancer cells. Note that there was no change in the level of total Akt in I3C-treated PC-3 cells. (C: control; 30, 60: 30, 60 μ M I3C)

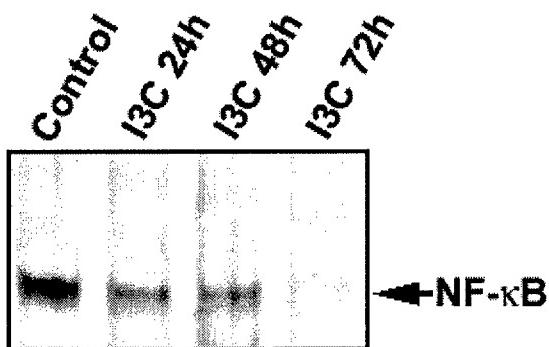


Figure 4: Gel mobility shift assay showing that the NF- κ B DNA binding activity is significantly inhibited by the treatment of 60 μ M I3C for 72 hours.

To investigate the relationship between Akt and NF- κ B in I3C treated PC-3 cells, we have conducted transfection experiments with Akt (wild, mutant, or constitutively activated) expression vector and NF- κ B-Luc vector that contains NF- κ B binding and reporter sequences, and measured the activity of luciferase as well as NF- κ B DNA binding activity in transfected PC-3 prostate cancer cells. We have detected the effects of I3C on Akt and NF- κ B in transfected and un-transfected PC-3 prostate cancer cells. We found that Akt transfection up-regulated activity of NF- κ B as demonstrated by luciferase assay (Figure 5) and EMSA (Figure 6). We also found that I3C inhibited the activation of NF- κ B induced by Akt transfection (Figure 5 and 6), suggesting that the inhibition of NF- κ B by I3C is partly mediated by Akt pathway.

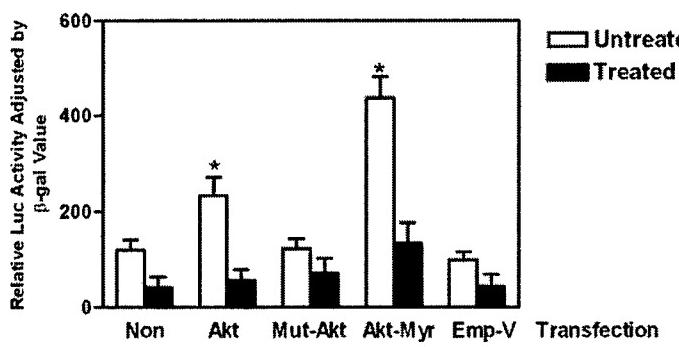


Figure 5: Luciferase activity in transfected PC-3 cells with or without 60 μ M I3C treatment. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-V: transfected with empty vector; *: $p < 0.05$; $n=2$)

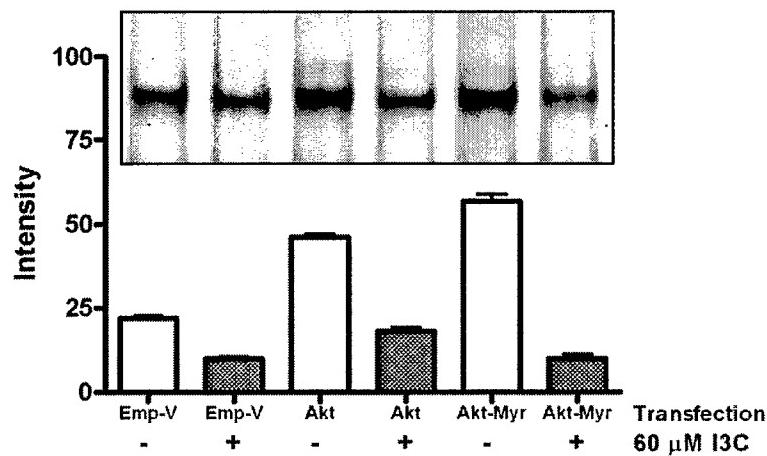


Figure 6: EMSA and densitometric analysis of NF- κ B DNA-binding activity in transfected PC-3 cells with or without I3C treatment. (Emp-V: transfected with empty vector; Akt: transfected with wild Akt; Akt-Myr: transfected with constitutively activated Akt.)

We have also investigated the pro-apoptotic effect of I3C in PC-3 prostate cancer cells and Akt transfected PC-3 cells. We found that I3C induced apoptosis in both parental and Akt transfected PC-3 cancer cells (Figure 7). However, Akt transfection showed inhibitory effect on the apoptosis induced by I3C, suggesting that the pro-apoptotic effect of I3C is mediated by Akt and NF- κ B pathways.

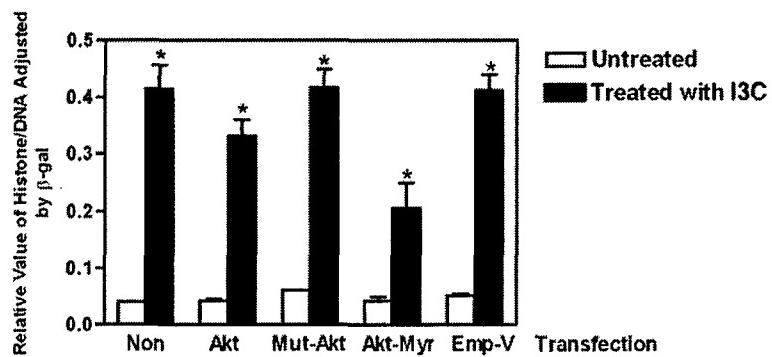


Figure 7: Induction of apoptosis in 60 μ M I3C treated PC-3 cells tested by ELISA. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-v: transfected with empty vector; *: $p < 0.05$; $n=2$)

The activation of Akt and NF- κ B is believed to be responsible for the resistance to chemotherapeutic agents, which is the major cause for treatment failure in cancer chemotherapy. Inhibition of NF- κ B or Akt may potentiate the anticancer effect of chemotherapeutic agents. We have found that I3C inhibited the activation of Akt and NF- κ B and induced apoptosis, suggesting that I3C can sensitize cancer cells to apoptotic death induced by chemotherapeutic agents. By cell growth inhibition assay, we found that combination treatment with I3C and cisplatin significantly inhibited the growth of PC-3 prostate cancer cells compared to monotreatment (Figure 8). These results suggest that I3C can potentiate the anticancer effect of chemotherapeutic agents through inactivation of Akt and NF- κ B.

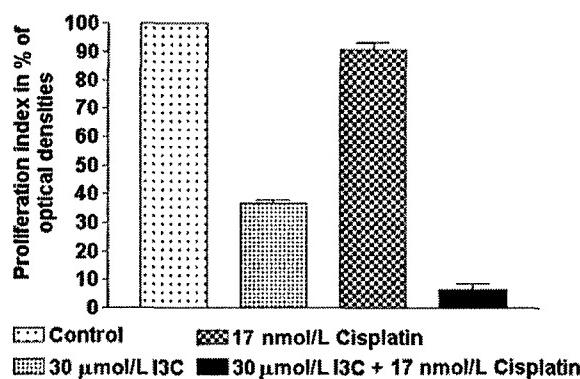


Figure 8. Growth inhibition of PC-3 prostate cancer cells exposed to I3C for 24h and then cisplatin for 48 h. Combination treatment with I3C and cisplatin significantly inhibited the growth of PC-3 cells compared to monotreatment.

To investigate whether DIM also sensitizes prostate cancer cells to death induced by chemotherapeutic agents, we also tested the effects of combination treatment with DIM and Taxotere on the growth of PC-3 prostate cancer cells. We found that the combination treatment with DIM and Taxotere exerted significantly more growth inhibitory effect on PC-3 cells (Figure 9). We have also done similar experiment using androgen sensitive LNCaP cells which contains functional androgen receptor (AR) and also secretes PSA. The results of our study have been

presented in 96th AACR Annual Meeting (9; also see Appendices 2). The summary of our results in the form of an abstract is presented below.

Abstract: Despite initial response to androgen deprivation, patients with prostate cancer usually develop hormone refractory metastasis and eventually succumb to the disease. Therefore, new therapeutic strategies are urgently needed to overcome drug resistance for the treatment of prostate cancer. Epidemiological studies have shown that the consumption of cruciferous vegetables is associated with reduced cancer risk. 3,3'-diindolymethane (DIM) is an acid condensation product of I3C, which is present in almost all members of the cruciferous vegetable family. DIM has been shown to down-regulate AR, suggesting the growth inhibitory effects of DIM on hormone related cancers. A formulated DIM (B-DIM, BioResponse) has been shown to exhibit approximately 50% higher bioavailability. However, no molecular studies have been reported to date to elucidate the effect of B-DIM on prostate cancer cells. To investigate the effects of B-DIM on prostate cancer cells, we treated hormone sensitive LNCaP cells and hormone insensitive PC-3 cells with 0 to 60 μ M of B-DIM for one to three days. MTT, ELISA, and Western blot analysis were conducted to test cell growth inhibition, apoptosis, and the expression of AR and prostate specific antigen (PSA). Microarray analysis was also conducted to assess global alterations in gene expression profile. We found that B-DIM inhibited the growth of prostate cancer cells and induced apoptosis in a dose and time dependent manner. Microarray gene expression analysis showed that B-DIM regulated the expression of genes which are critical for the control of cell growth and apoptosis. Western blot analysis showed that B-DIM significantly inhibited AR and PSA protein expression in LNCaP prostate cancer cells. It has been recently reported that increases in androgen receptor mRNA and proteins are both necessary and sufficient to convert prostate cancer from a hormone-sensitive to a hormone refractory stage, suggesting that over-expression of AR is an important factor for acquired resistance of prostate cancer to hormone ablation therapy. Since our results showed decreased AR and PSA expression with concomitant inhibition of cell growth and induction of apoptosis by B-DIM, we suggest that the resistance acquired by prostate cancer cells to androgen ablation therapy could be reversed by B-DIM treatment. Moreover, we tested our hypothesis whether B-DIM could sensitize hormone insensitive PC-3 prostate cancer cells to Taxotere. We found that Taxotere (1 nM) in combination with 50 μ M B-DIM exerted significantly more growth inhibitory effects compared to Taxotere or B-DIM treatment alone. In conclusion, our results provide a solid foundation for devising novel therapeutic strategies for the treatment of hormone or chemo-resistant metastatic prostate cancer by including B-DIM, a non-toxic agent, with other therapeutic agents.

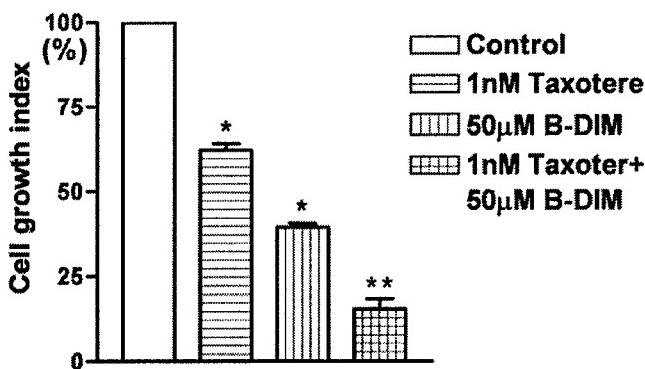


Figure 9. Growth inhibition of PC-3 prostate cancer cells exposed to DIM and Taxotere for 72h significantly inhibited the growth of PC-3 cells compared with monotreatment (*: $p<0.05$; **: $P<0.01$).

We also investigated the effect of I3C on the expression of genes related to the control of cell growth, apoptosis, and Akt and NF- κ B signal pathways. The results of our study have been published in "The Journal of Nutrition" (10; also see Appendices 3). The summary of our results in the form of an abstract is presented below.

Abstract: Epidemiological and dietary studies have shown an association between high dietary intake of cruciferous vegetables and decreased prostate cancer risk. Our studies have shown that indole-3-carbinol (I3C), a common phytochemical in cruciferous vegetable, and its *in vivo* dimeric product 3,3'-diindolylmethane (DIM) up-regulate the expression of phase I and phase II enzymes, suggesting increased capacity for detoxification and inhibition of carcinogens. Studies from our laboratory and others have found that I3C could induce cell cycle arrest in G1 phase by up-regulation of p21^{WAF1} and p27^{KIP1}, and down-regulation of CDK6 in PC-3 prostate cancer cells. I3C treatment also induced apoptosis with down-regulation of Bcl-2 and Bcl_{XL}, up-regulation of Bax, translocation of Bax to mitochondria, and cytochrome c release. In addition, we found by microarray gene expression profiling that I3C/DIM regulates many genes that are important for the control of cell cycle, cell proliferation, signal transduction and other cellular processes, suggesting the pleiotropic effects of I3C/DIM on prostate cancer cells. Moreover, we have recently found that I3C functions as an inhibitor of Akt and NF- κ B, which play important roles in cell survival, and are believed to be potential targets in cancer prevention and therapy. We have also shown previously that forced over-expression of Akt in prostate cancer cells by Akt gene transfection, leads to the activation of NF- κ B and this process could be abrogated by I3C/DIM treatment, suggesting that I3C could be a potent agent not only for the induction of cell death but also may sensitize prostate cancer cells to common chemotherapeutic agent. Studies have already shown that the inactivation of Akt and NF- κ B is responsible for chemo-sensitization of chemo-resistant cancer cells. Since there is no effective treatment strategy for hormone dependent, but most importantly, hormone independent and metastatic prostate cancer, our strategies to sensitize prostate cancer cells to chemotherapeutic agent induced killing of prostate cancer cells by I3C/DIM is a novel breakthrough, which could be used for devising novel therapeutic strategies for prostate cancer. In conclusion, the results from our laboratory and others provide ample evidence for the benefit of I3C/DIM for prevention and/or treatment of prostate cancer.

Our published review article (11; also see Appendices 4) is also attached to provide comprehensive results that we have obtained thus far.

Conclusion: The task-2 has been fully completed.

Task-3. Our task 3 was to determine whether treatment of prostate cancer cells with I3C and DIM will elicit responses that may determine causal association between the inactivation of Akt and NF- κ B with the induction of apoptosis. We investigated different kinases that are involved in the NF- κ B pathway, particularly the status of IKK, MAP kinase-MEK, MEKK1 and NIK. In order to establish cause and effect relationships of these genes with the ultimate effect of I3C and DIM, we are now conducting several transfection experiments which are very time consuming and labor intensive. We have spent a lot of time to standardize the optimal conditions for transfection experiments. We have tested the transfection efficacy using IKK α , IKK β , MEKK, and NIK cDNA plasmids so far. We have measured the expression of IKK α , IKK β , MEKK, and NIK after transfections with these plasmids using Western Blot analysis. We have also measured the expression of IkB α , phosphor-

I κ B α , and NF- κ B in some samples which were transfected with IKK α , IKK β , MEKK, and NIK, and subsequently treated with or without I3C and DIM. By electrophoretic mobility shift assay (EMSA), we have also tested the DNA binding activity of NF- κ B in some samples which were transfected with IKK β and IKK α , and subsequently treated with or without I3C and DIM. Our findings from these experiments are presented below.

A. IKK β transfection.

In human cells, NF- κ B is sequestered in the cytoplasm through tight association with its inhibitory protein, I κ B α . The activation of NF- κ B occurs through site-specific phosphorylation and ubiquitination of I κ B α by I κ B kinase β (IKK β). I κ B α is subsequently degraded by the 26S proteasome. NF- κ B becomes free from I κ B α and translocates into nucleus for binding to NF- κ B-specific DNA-binding sites, regulating target gene transcription (Diagram 1). Because IKK β is the important molecule which regulates the activity of NF- κ B, we have investigated the effects of I3C and DIM on IKK-NF- κ B pathway by transfection of IKK β into prostate cancer cells.

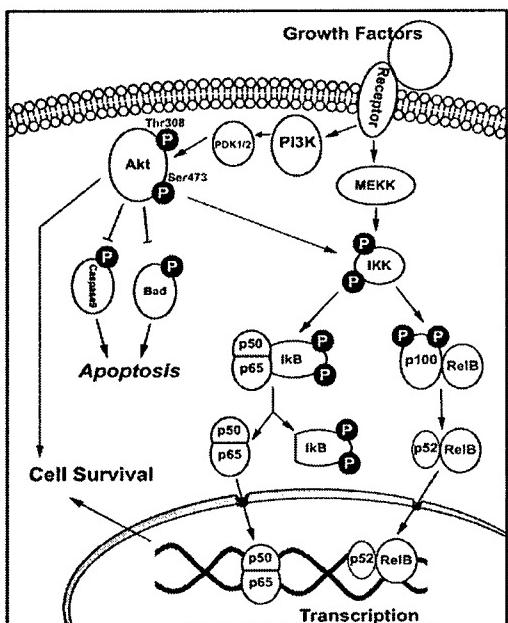


Diagram 1: Regulation of NF- κ B and Akt signaling pathways.

We have transfected IKK β cDNA into PC-3 prostate cancer cells. The IKK β transfected cells were treated with or without 60 μ M I3C or 40 μ M DIM. We found that the expression of IKK β was significantly up-regulated with IKK β cDNA transfection. The transfection efficacy was equal in all transfection samples because the equal expression of FLAG, which was expressed by the transfected plasmids, was observed. We also found that I3C or DIM inhibited the expression of IKK β in PC-3 prostate cancer cells, similar to another chemopreventive agent, genistein (Figure 10).

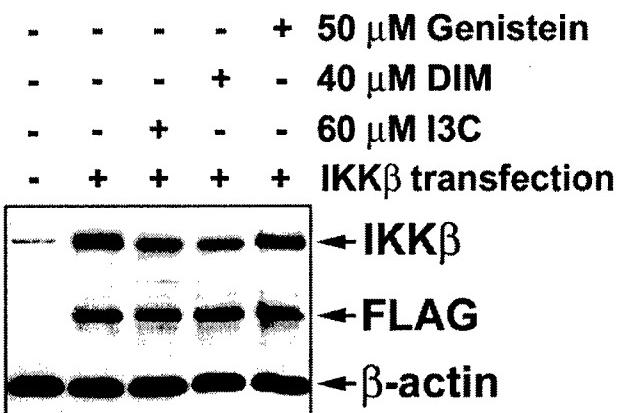


Figure 10. Western Blot analysis showed that expression of IKK β was significantly up-regulated with IKK β cDNA transfection and was down-regulated by I3C and DIM treatment. Similar results were also seen with genistein.

Since we observed the down-regulation of IKK β by I3C and DIM, we tested the levels of phospho-I κ B α protein, which has been known to be produced from phosphorylation of I κ B α by IKK β . We found more phospho-I κ B α protein in IKK β transfected PC-3 cells compared to parental cells. Importantly, significant down-regulation of phosphorylated I κ B α was observed in IKK β transfected cells treated with I3C or DIM, suggesting that the inhibition of I κ B α phosphorylation by I3C or DIM may be mediated by the down-regulation of IKK β (Figure 11).

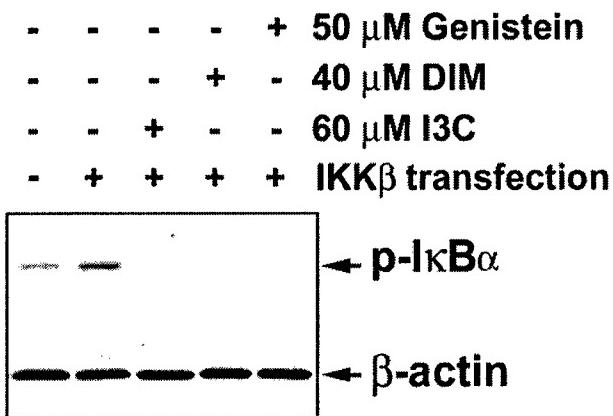


Figure 11. Western Blot analysis showed that expression of phospho-I κ B α was up-regulated with IKK β cDNA transfection and was significantly down-regulated by I3C and DIM treatment. Similar results were also seen with genistein.

We also tested the level of I κ B α protein. We found that the transfection of IKK β caused a slight decrease in the level of I κ B α . However, I3C and DIM treatment increased the level of I κ B α protein, suggesting the lower degree of I κ B α phosphorylation after I3C and DIM treatment (Figure 12), and these results are consistent with results presented in Figure 11.

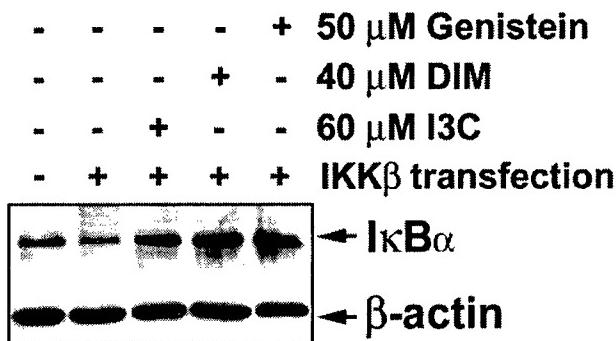
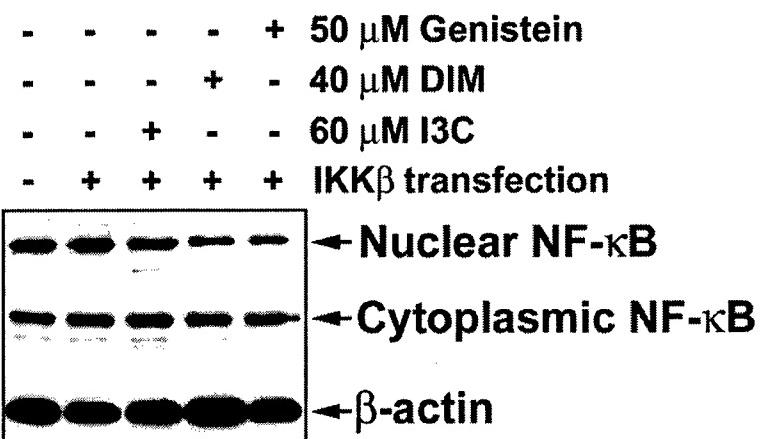
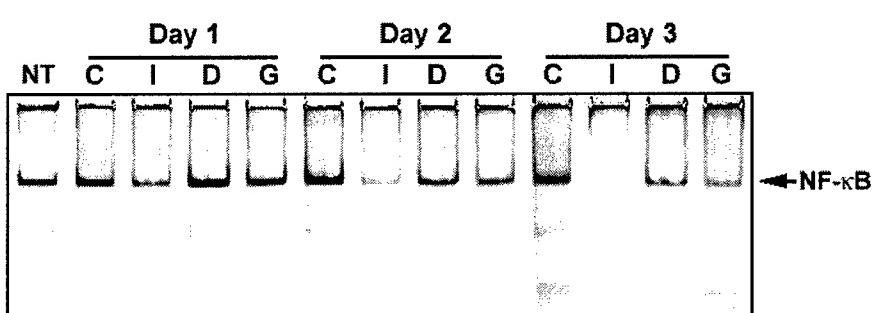


Figure 12. Western Blot analysis showed that expression of I κ B α was down-regulated with IKK β cDNA transfection and was up-regulated by I3C and DIM treatment. Similar results were also seen with genistein.

Because we observed the inhibitory effects of I3C and DIM on the I κ B α phosphorylation that activates NF- κ B, we measured the levels of NF- κ B protein in cytoplasmic and nuclear extracts, respectively. We found that the level of cytoplasmic NF- κ B remained unchanged upon I3C or DIM treatment. However, the level of nuclear NF- κ B was significantly decreased after I3C or DIM treatment, suggesting that I3C and DIM inhibited the nuclear localization of NF- κ B (Figure 13).



We also tested the DNA binding activity of nuclear NF-κB by EMSA. We found that I3C and DIM inhibited the DNA binding activity of nuclear NF-κB in a time-dependent manner in IKK β transfected PC-3 cells, consistent with our Western Blot data (Figure 14).



with 40 μ M DIM; G: treated with 50 μ M genistein).

These results suggest that I3C and DIM may inhibit the expression of IKK β and the subsequent phosphorylation of I κ B α , resulting in the inhibition of nuclear localization and activation of NF-κB. These effects of I3C and DIM may be causally related to the inhibition of cancer cell growth and the induction of apoptosis as observed in our earlier results.

B. IKK α transfection.

The activation of NF-κB also occurs through phosphorylation of p100 by IKK α , leading to the processing of p100 into small form (p52). This process allows NF-κB (p52-RelB) to transfer into the nucleus for binding to NF-κB-specific DNA-binding sites and, in turn, regulating gene transcription (Diagram 1). Because of the importance of IKK α in the NF-κB pathway, we have also transfected IKK α cDNA into PC-3 prostate cancers. The IKK α transfected cells were treated with or without 60 μ M I3C or 40 μ M DIM. We found that the expression of IKK α was significantly up-regulated with IKK α cDNA transfection. We also found that DIM inhibited the expression of IKK α in PC-3 prostate cancer cells, but no significant effect was observed with I3C (Figure 15).

Figure 13. Western Blot analysis showed that expression of nuclear NF-κB was up-regulated with IKK β cDNA transfection and was down-regulated by I3C and DIM treatment. The level of cytoplasmic NF-κB remained unchanged. Similar results were also seen with genistein.

Figure 14. EMSA showed that the DNA binding activity of NF-κB was down-regulated by I3C and DIM treatment in IKK β transfected cells (NT: no transfection; C: control; I: treated with 60 μ M I3C; D: treated

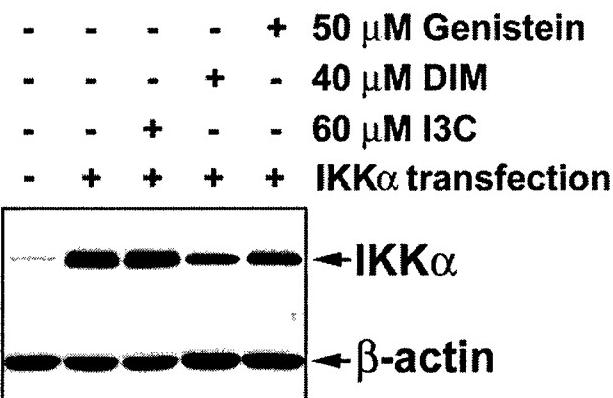


Figure 15. Western Blot analysis showed that expression of IKK α was significantly up-regulated with IKK α cDNA transfection and was down-regulated by DIM treatment but I3C appears to be ineffective. Similar results were also seen with genistein.

C. MEKK transfection.

It has been known that IKK could be phosphorylated and activated by an upstream kinase, mitogen activated kinase kinase 1 (MEKK1). Therefore, MEKK is another important molecule which regulates the activity of NF- κ B (Diagram 1). Hence, we have transfected MEKK cDNA into PC-3 prostate cancers. The MEKK transfected cells were treated with or without 60 µM I3C or 40 µM DIM. We found that the expression of IKK β was up-regulated with MEKK cDNA transfection. We did not observe significant alteration in the expression of MEKK in I3C or DIM treated PC-3 prostate cancer cells (Figure 16), suggesting that MEKK is not a target of I3C or DIM. Similarly, MEKK is not a clear target of genistein, which was used as a control.

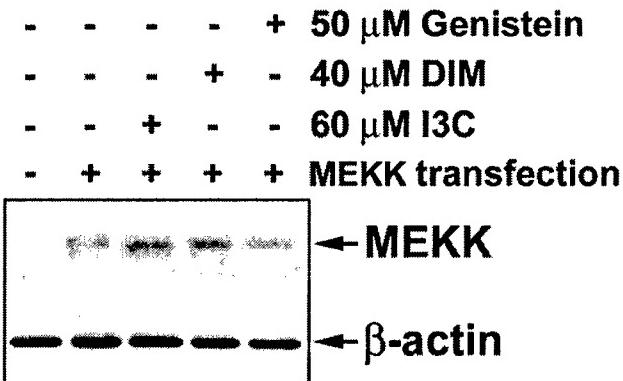


Figure 16. Western Blot analysis showed that expression of MEKK was up-regulated with MEKK cDNA transfection. No significant alteration in the expression of MEKK was observed by I3C and DIM treatment in MEKK transfected PC-3 cells.

Conclusion: We have completed certain parts of task-3 and plan to complete this task in the third year.

Task-4. Once the tasks-3 is completed, we will make progress on task-4 to complete all experiments, data analysis and manuscript writing during the third year of funding.

Key Research Accomplishments

- Demonstrated the effect of I3C on NF-κB DNA binding activity in PC-3 prostate cancer cells by EMSA.
- Demonstrated the effects of I3C on phosphorylation of Akt in PC-3 prostate cancer cells by Western blot analysis.
- Demonstrated the cross-talk between Akt and NF-κB in PC-3 prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the effects of I3C on Akt and NF-κB pathways in Akt transfected PC-3 prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the effects of I3C on the induction of apoptosis in Akt transfected PC-3 prostate cancer cells by transfection and ELISA.
- Determined the effects of I3C and DIM on the sensitization of prostate cancer cells to cell death induced by chemotherapeutic agents, cisplatin and Taxotere.
- Conducted IKK β gene transfection study and investigated the effects and the molecular mechanisms of I3C and DIM action on the regulation of NF-κB pathway.
- Conducted IKK α gene transfection study and investigated the effects and the molecular mechanisms of I3C and DIM action on the regulation of NF-κB pathway.
- Conducted MEKK and NIK gene transfection studies and investigated the effects of I3C and DIM on the regulation of NF-κB pathway.

Reportable Outcomes

1. Li Y, Chinni SR, Sarkar FH. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by Akt and NF-κB pathways in prostate cancer cells. *Front Biosci* 10:236-243, 2005
2. Sarkar FH, Bhuiyan MM, Pilder M, Li Y. Down regulation of androgen receptor (AR) and its target gene (PSA) expressions by B-DIM leads to sensitization of prostate cancer cells to Taxotere-induced apoptosis. *Proc Am Assoc Cancer Res* 46:abstr 466, 2005.
3. Sarkar FH, Li Y. Indole-3-Carbinol and Prostate Cancer. *J Nutr* 134:3493S-3498S, 2004.
4. Sarkar FH, Li Y. Cell signaling pathways altered by natural chemopreventive agents. *Mutat Res* 555:53-64, 2004.

Conclusions

We have found that I3C and DIM inhibit the phosphorylation of Akt and the activity of Akt in prostate cancer cells. We have also found that I3C and DIM down-regulate NF-κB DNA binding activity in prostate cancer cells. The inhibition of NF-κB by I3C and DIM is partly mediated through the inactivation of Akt. I3C and DIM inhibit phosphorylation of Akt, inactivate NF-κB, and regulate the expression of genes related to the control of cell growth and apoptosis, resulting in inhibition of cell growth and induction of apoptosis in prostate cancer cells. We have also found that I3C and DIM inhibited the expression of IKK β , the subsequent phosphorylation of I κ B α , and the nuclear localization and activation of NF-κB, resulting in the inhibition of cell growth and induction of apoptosis in prostate cancer cells. These results along with our previous findings suggest that I3C or DIM could be potent agent for the prevention and/or treatment of prostate cancer. The task-1 and task-2 have been fully completed. We have completed certain parts of task-3. We plan to complete task-3 and task-4 in the third year as originally planned.

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11. Sarkar FH, Li Y. Cell signaling pathways altered by natural chemopreventive agents. *Mutat Res* 555: 53-64, 2004.

Appendices

Publications and abstracts during the second year of funding:

1. Li Y, Chinni SR, Sarkar FH. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by Akt and NF- κ B pathways in prostate cancer cells. *Front Biosci* 10:236-243, 2005.
2. Sarkar FH, Bhuiyan MM, Pilder M, Li Y. Down regulation of androgen receptor (AR) and its target gene (PSA) expressions by B-DIM leads to sensitization of prostate cancer cells to Taxotere-induced apoptosis. *Proc Am Assoc Cancer Res* 46:abstr 466, 2005.
3. Sarkar FH, Li Y. Indole-3-Carbinol and Prostate Cancer. *J Nutr* 134:3493S-3498S, 2004.
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SELECTIVE GROWTH REGULATORY AND PRO-APOPTOTIC EFFECTS OF DIM IS MEDIATED BY AKT AND NF-kappaB PATHWAYS IN PROSTATE CANCER CELLS

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1. ABSTRACT

Prostate cancer is the second leading cause of cancer related deaths in men in the United States. I3C and its *in vivo* dimeric product, DIM, have been found to inhibit the growth of prostate cancer cells. However, the molecular mechanism(s) by which DIM elicits its effects on prostate cancer cells has not been fully elucidated. We have previously shown that I3C induces apoptosis and inhibits the activation of NF-kappaB pathway, which could be mediated via Akt signaling pathway. In this study, we investigated whether there is any cross-talk between Akt and NF-kappaB during DIM-induced apoptosis in PC-3 prostate cancer cells. We found that DIM inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells but not in non-tumorigenic CRL2221 human prostate epithelial cells. DIM also inhibited EGFR expression, PI3K kinase activity, and Akt activation, and abrogated the EGF-induced activation of PI3K in prostate cancer cells. NF-kappaB DNA-binding analysis and transfection studies with Akt cDNA constructs revealed that Akt transfection resulted in the induction of NF-kappaB activity and this was inhibited by DIM treatment. DIM treatment also showed significant induction of apoptosis in non-transfected cells compared to Akt and Akt-Myr transfected prostate cancer cells. From these results, we conclude that the inhibition of Akt and NF-kappaB activity and their cross-talk is a novel mechanism by which DIM inhibits cell growth and induces apoptotic processes in prostate cancer cells but not in non-tumorigenic prostate epithelial cells.

2. INTRODUCTION

Prostate cancer is one of the most common cancers in men and the second leading cause of male cancer death in the United States (1). However, Asians have relatively low incidence of prostate cancers. Dietary and epidemiological studies have shown an association between high dietary intake of vegetables and decreased prostate cancer risk (2, 3). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family including broccoli, cabbage, brussels sprouts, and cauliflower appears to be most effective at reducing the risk of cancers. Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family, and it is readily converted to its dimeric product, 3,3'-diindolylmethane (DIM) (4). There are growing evidences showing that I3C and DIM have the potential to inhibit a number of common cancers, especially those that are hormone-related (5-8).

It has been demonstrated that I3C and DIM possess anti-carcinogenic effects in experimental animals and inhibits the growth of human cancer cells *in vitro* (5, 7-10). DIM has been found to induce cell cycle arrest at G1 phase with up-regulation of p21^{WAF1} and down-regulation of CDK6 (6, 11). It has been reported that DIM increases the expression of Bax, decreases the expression of Bcl-2, and induces apoptosis (12). Because of these effects, the interest in I3C and DIM as cancer chemopreventive and/or

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therapeutic agents has significantly increased in the past years. We have previously shown that I3C up-regulates p21^{WAF1}, Bax, and p27^{KIP1}, and down-regulates Bcl_{XL}, EGFR, and Akt kinase activity, leading to the induction of apoptosis in prostate cancer cells (13, 14). We have also reported the gene expression profiles of prostate cancer cells exposed to I3C and DIM, showing that I3C and DIM induce the expression of genes related to the Phase I and Phase II enzymes and regulate the expression of genes involved in the control of cell growth, cell cycle, apoptosis, signal transduction, and oncogenesis (15). However, the precise molecular mechanism by which DIM exerts its effect on the induction of apoptosis and the cell signaling pathways, has not been fully elucidated.

Akt and NF-kappaB pathways are important cell signaling pathways involved in the processes of apoptosis, carcinogenesis, and tumor progression (16-19). NF-kappaB is a cell survival factor and can be activated by many types of stimuli including TNF- α , EGF, UV radiation, etc. There is growing evidence to suggest the role of NF-kappaB in the protection against apoptosis (18, 20). An *in vivo* study showed that mice lacking NF-kappaB p65/RelA died embryonically from extensive apoptosis in the liver (21), suggesting anti-apoptotic role of NF-kappaB. Akt can be activated by various growth factors including EGF through activation of phosphatidylinositol-3 kinase (PI3K) (17). Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate downstream targets (16, 17). Several reports have showed that Akt also regulates the NF-kappaB pathway via phosphorylation and activation of molecules in the NF-kappaB pathway (22-24). Because both Akt and NF-kappaB have been critically involved in the cell survival and apoptotic process, in this study, we investigated whether DIM could inhibit Akt and NF-kappaB activation leading to apoptosis, and whether Akt and NF-kappaB pathways could cross-talk during apoptotic process induced by DIM. Finally we also investigated whether there is any differential effect of DIM between PC-3 prostate cancer cells and non-tumorigenic CRL2221 prostate epithelial cells.

3. MATERIALS AND METHODS

3.1. Cell culture and reagents

PC-3 human prostate cancer cells (ATCC, Manassas, VA) were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO₂ atmosphere at 37°C. CRL-2221 human non-tumorigenic prostate epithelial cells (ATCC, Manassas, VA) were cultured in keratinocyte-SFM media (Invitrogen, Carlsbad, CA) supplemented with EGF (0.2 microgram/L), bovine pituitary extract (30 milligram/L), and 1% penicillin and streptomycin. DIM (LKT, St. Paul, Minnesota) was dissolved in DMSO to make 60 millimole/L stock solution. Wherever indicated, EGF (Invitrogen, Carlsbad, CA) was added to the media at a final concentration of 100 microgram/L.

3.2. Cell growth inhibition by MTT assay

The PC-3 and CRL-2221 cells were seeded at a density of 1×10³/well in 96 well culture dishes. After 24 hours, the cells were treated with 15, 30, and 60 micromole/L DIM for one to three days. Control PC-3 cells received 0.1% DMSO for same time points. The cells were then incubated with MTT (0.5 gram/L, Sigma, St. Louis, MO) at 37°C for 4h and with DMSO at room temperature for 1 h. The spectrophotometric absorbance of the samples was measured by using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 595 nm. The experiment was repeated three times and t test was performed to verify the significance of cell growth inhibition after treatment.

3.3. Histone/DNA ELISA for detecting apoptosis

Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA, USA) was used to detect apoptosis in PC-3 and CRL-2221 cells with different treatments according to manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from PC-3 and CRL-2221 cells treated with 15, 30, and 60 micromole/L DIM or 0.1% DMSO (vehicle control) for 24, 48, 72 hours, were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 405 nm.

3.4. Western blot analysis

The PC-3 and CRL-2221 cells were plated on culture dishes and allowed to attach for 24 hours followed by the treatment with 15, 30, or 60 micromole/L DIM for 48 hours. Control cells were incubated in the medium with 0.1% DMSO using same time points. After incubation, the cells were lysed in 62.5 millimole/L Tris-HCl and 2% SDS. Protein concentration was then measured using BCA protein assay (PIERCE, Rockford, IL). Cell extracts were subjected to 10% SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. Membranes were incubated with anti-Akt (Santa Cruz Biotech, Santa Cruz, CA), anti-phospho-Akt Ser473 (Cell signaling, Beverly, MA), anti-EGFR (Santa Cruz Biotech, Santa Cruz, CA), anti-Bcl_{XL} (Santa Cruz Biotech, Santa Cruz, CA), and anti-β-actin (Sigma, St. Louis, MO) antibodies, washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (PIERCE, Rockford, IL).

3.5. Reporter gene constructs and transfection

pLNCX-Akt (normal Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control vector) were generously provided by Dr. Sellers (Dana-Farber Cancer Institute, Boston, MA). NF-kappaB-LUC (Stratagene, La Jolla, CA) contains six repeated copies of the NF-kappaB DNA-binding site and a luciferase reporter gene. CMV-beta-gal reporter construct transfection was used for normalization of transfection efficiency. The pLNCX-Akt,

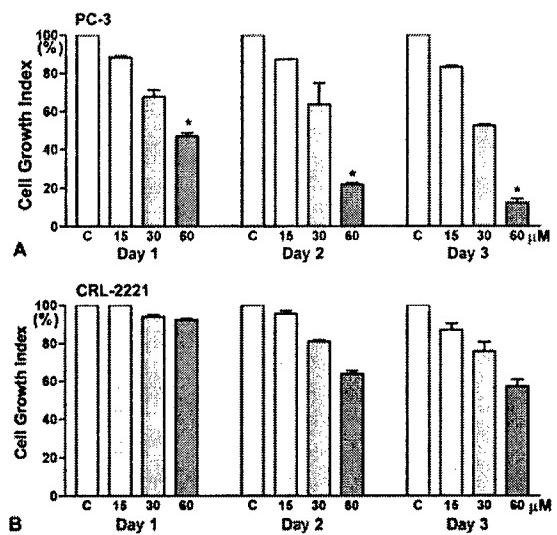


Figure 1. Effects of DIM on the growth of PC-3 (A) and CRL-2221 cells (B) tested by MTT assay. (*: $p<0.05$; n=3).

pLNCX-Myr-Akt, pLNCX-Akt-K179M, or pLNCX was transiently co-transfected with NF-kappaB-LUC and CMV-beta-gal into PC-3 cells when they were at ~70% confluent using the LipofectAMINE (Invitrogen, Carlsbad, CA). After incubation for 5 hours, the transfected cells were washed and incubated with RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum overnight followed by treatment with 60 micromole/L DIM for 48 hours. Subsequently, the luciferase activities in the samples were measured by using Steady-GloTM Luciferase assay system (Promega, Madison, WI) and ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC). To detect the NF-kappaB activity in transfected PC-3 cells, the samples were subjected to NF-kappaB DNA-binding activity measurement using EMSA method as described below. Cell Apoptosis ELISA Detection Kit was also used to detect apoptosis in transfected and parental PC-3 cells.

3.6. NF-kappaB DNA-binding activity measurement

PC-3 and CRL-2221 cells were treated with 15, 30, and 60 micromole/L DIM for 72 hours. Following treatment, the nuclear proteins from cells were extracted. Ten microgram of nuclear proteins was subjected to electrophoretic mobility shift assay (EMSA) as described previously (25). Competition assay using unlabeled specific competitor (NF-kappaB oligo) was conducted to confirm the specificity of NF-kappaB DNA-binding activity.

3.7. Immunoprecipitation and PI3K kinase assay

The PI3K kinase activity of PC-3 cells treated with 60 micromole/L DIM for 24 and 48 hours, 60 micromole/L DIM for 24 and 48 hours followed by EGF treatment for 20 minutes, EGF only, or 0.1% DMSO was measured by using PI3K kinase assay kit (Echelon, Salt Lake, UT) according to manufacturer's protocol. Briefly, the cells after treatments were lysed in ice-cold cell lysis buffer (137 millimole/L NaCl, 20 millimole/L Tris-HCl, 1

millimole/L CaCl₂, 1 millimole/L MgCl₂, 0.1 millimole/L sodium orthovanadate, 1% NP-40, and 1 millimole/L PMSF) on ice for 20 minutes. After centrifugation, the protein concentration of supernatant was measured by BCA protein assay (PIERCE, Rockford, IL). 600 micrograms of proteins from each sample were used for immunoprecipitation with PI3K antibody (Upstate, Charlottesville, VA) overnight and protein G-agarose for one hour at 4°C. Then, the samples were collected by centrifugation, washed with kinase buffer, and subjected to PI3K kinase assay in kinase buffer (20 millimole/L Tris pH 7.4, 4 millimole/L MgCl₂, 10 millimole/L NaCl), 25 micromole/L ATP, and 2.4 micrograms of PI(4,5)P₂ as kinase substrate. PI(3,4,5)P₃ was measured by competitive ELISA.

3.8. Signal quantification and statistical analysis

The EMSA gel was scanned, and the signals in the gel were quantified and analyzed with Odyssey software (LI-COR, Lincoln, NE). Signal in the Western blots was also scanned and quantified with Molecular Analyst software (Bio-Rad, Hercules, CA). Comparisons were made between control and treatments. Statistical analysis was performed using t test between treated and untreated samples. P values less than 0.05 were used to indicate statistical significance.

4. RESULTS

4.1. DIM selectively inhibits growth of prostate cancer cells

PC-3 prostate cancer cells and CRL-2221 non-tumorigenic human prostate epithelial cells were treated with 0-60 micromole/L DIM over 3 days and the cell viability was determined by MTT assay. The treatment of PC-3 prostate cancer cells with DIM resulted in a dose and time-dependent inhibition of cell proliferation (Figure 1A). However, only 37.8% growth inhibition was observed in the CRL-2221 non-tumorigenic prostate epithelial cells treated with 60 micromole/L DIM for 3 days compared to 87.8% in PC-3 cells (Figure 1B), suggesting the selective growth inhibition of prostate cancer cells by DIM. Inhibition of cell proliferation observed by MTT could be partly due to the induction of apoptosis in prostate cancer cells. We, therefore, investigated whether DIM could selectively induce apoptosis in PC-3 prostate cancer cells.

4.2. DIM selectively induces apoptosis in prostate cancer cells

By ELISA analysis of cytoplasmic histone/DNA fragments, we observed an induction of apoptosis in prostate cancer cells treated with 15-60 micromole/L DIM (Figure 2A). The induction of apoptosis was time- and dose-dependent, and was directly correlated with the inhibition of cell growth, suggesting that DIM treatment may result in the inhibition of cell proliferation through apoptotic cell death. More importantly, non-tumorigenic CRL-2221 prostate epithelial cells were much less responsive to DIM treatment than PC-3 cells (Figure 2B), suggesting that DIM selectively induced apoptosis in prostate cancer cells.

Regulation of Akt & NF-kappaB pathways by DIM

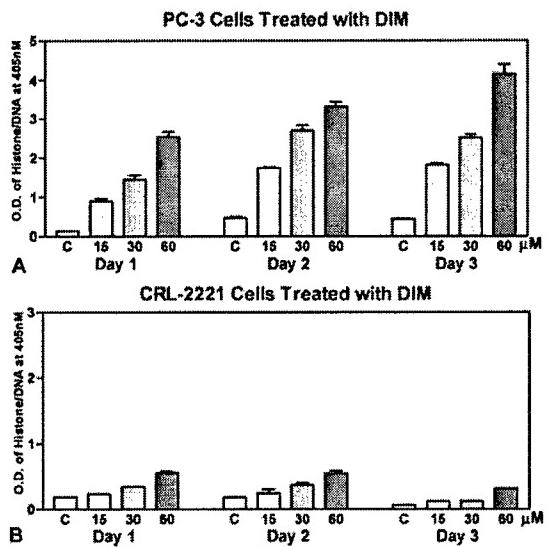


Figure 2. Induction of apoptosis in PC-3 (A) and CRL-2221 (B) cells tested by ELISA. (n=2).

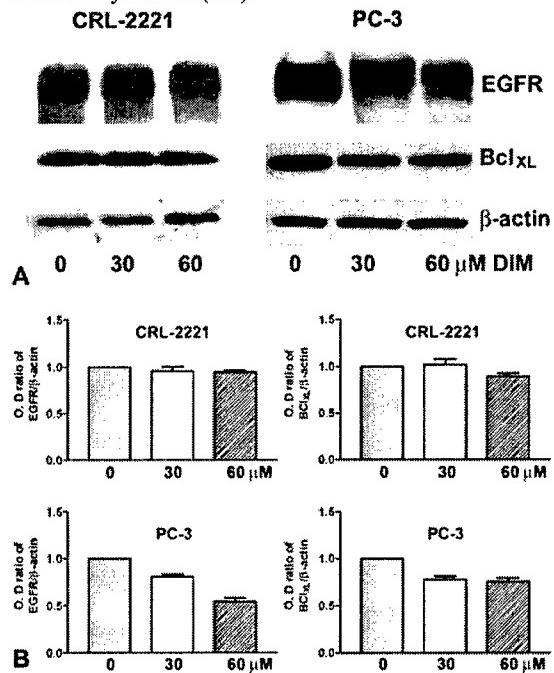


Figure 3. Western blot (A) and densitometric analysis (B) of Bcl_{XL} and EGFR in PC-3 and CRL-2221 cells treated with DIM.

By Western Blot analysis, we also found that DIM inhibited the expression of Bcl_{XL}, an anti-apoptotic protein in PC-3 cells (Figure 3). However, there was no significant effect on Bcl_{XL} in DIM-treated CRL-2221 cells, and these results were correlated with minimal apoptosis in DIM-treated CRL-2221 cells. Next, we investigated whether PI3K/Akt and NF-kappaB signaling pathways are involved in the apoptotic processes induced by DIM in prostate cancer cells.

4.3. DIM inhibits PI3K and Akt activation and induces apoptosis through Akt pathway

Since Akt signaling pathway is an important signal transduction pathway that plays a critical role in cell survival and apoptotic processes, we investigated the status of Akt in PC-3 and CRL-2221 cells treated with 0-60 micromole/L DIM by Western Blot analysis. We did not find any alterations in the protein expression of unphosphorylated Akt in DIM-treated PC-3 and CRL-2221 cells (Figure 4). However, a significant decrease in the phosphorylated Akt protein at Ser473 was observed in DIM treated PC-3 cells compared to control cells, suggesting inactivation of Akt kinase after DIM treatment (Figure 4). Treatment of DIM showed dose dependent inhibition of Akt phosphorylation in PC-3 cells, consistent with the induction of apoptosis by DIM. However, the phosphorylated Akt Ser473 protein was undetectable in CRL-2221 cells treated or untreated with DIM, suggesting that the inactivation of Akt by DIM is specific in prostate cancer cells compared to non-tumorigenic prostate epithelial cells.

Since Akt is activated through the activation of PI3K, we investigated the PI3K kinase activity in the PC-3 cells treated with DIM or pre-treated with DIM followed by EGF stimulation. We found that DIM treatment inhibited the activity of PI3K (Figure 5), suggesting that DIM could inactivate Akt through the inhibition of PI3K activity. We also found that EGF treatment alone activated PI3K kinase activity as expected, and that DIM pretreatment abrogated the activation of PI3K stimulated by EGF (Figure 5). Because the activation of Akt and PI3K could be mediated through EGFR pathways, we measured the expression of EGFR by Western Blot analysis. We found that DIM inhibited EGFR expression in a dose-dependent manner in PC-3 cells only and showed no inhibition of EGFR in CRL-2221 cells (Figure 3), corresponding with the selective effect of DIM on Akt activation in PC-3 cells.

Furthermore, we transfected Akt cDNA into PC-3 cells and measured the degree of apoptosis in transfected cells with and without DIM treatment. We found that DIM not only induced apoptosis in PC-3 parental cells but also in Akt transfected PC-3 cells although to a lesser extent as expected (Figure 6). More importantly, we found that transfection of constitutively activated Akt (pLNCX-Myr-Akt) and wild-type Akt (pLNCX-Akt) inhibited apoptosis induced by DIM compared to mutant Akt and empty vector transfecants, suggesting that the induction of apoptosis by DIM is partly mediated by active Akt and that the overexpression of Akt leads to resistance to DIM-induced apoptosis.

4.4. DIM selectively inhibits NF-kappaB activation in PC-3 cells

Nuclear extracts from control and DIM-treated PC-3 cells were subjected to NF-kappaB DNA-binding activity as measured by EMSA. Autoradiography revealed that 30-60 micromole/L DIM significantly inhibited NF-kappaB DNA-binding activity in PC-3 cells compared to untreated control (Figure 7). However, no such effect was observed in DIM-treated CRL-2221 cells, suggesting the

Regulation of Akt & NF-kappaB pathways by DIM

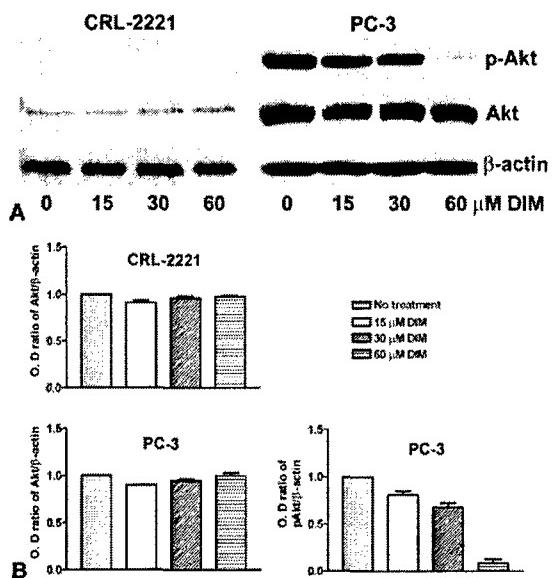


Figure 4. Western blot (A) and densitometric analysis (B) of total Akt and p-Akt in PC-3 and CRL-2221 cells treated with DIM.

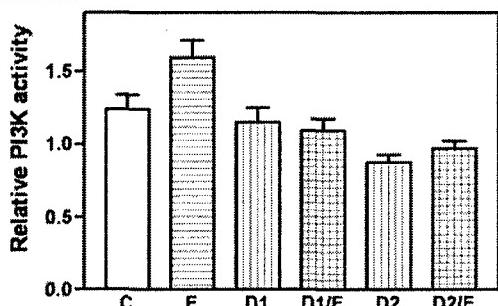


Figure 5. Relative PI3K activity in PC-3 cells treated with DIM. (C: control; E: treated with EGF; D1: treated with 60 micromole/L DIM for 24 hours; D1/E: treated with 60 micromole/L DIM for 24 hours followed by EGF treatment; D2: treated with 60 micromole/L DIM for 48 hours; D2/E: treated with 60 micromole/L DIM for 48 hours followed by EGF treatment).

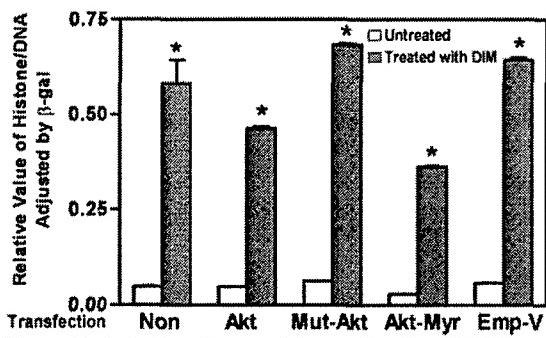


Figure 6. Induction of apoptosis in transfected and DIM treated PC-3 cells tested by ELISA (Non: no transfection; Akt: transfected with pLNCX-Akt; Mut-Akt: transfected with pLNCX-Akt-K179M; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

selective inhibitory effect of DIM on NF-kappaB DNA-binding activity in prostate cancer cells. In order to further explore the inhibitory effects of DIM on Akt and NF-kappaB pathways, we conducted transfection experiments as described under Materials and Methods. Luciferase assay showed a significant increase in luciferase activity in PC-3 cells co-transfected with pLNCX-Myr-Akt and NF-kappaB-Luc, and also in PC-3 cells co-transfected with pLNCX-Akt and NF-kappaB-Luc (Fig 8). Moreover, DIM significantly abrogated the induction of luciferase activity caused by pLNCX-Myr-Akt and pLNCX-Akt transfections (Figure 8).

To confirm these results, we also examined the NF-kappaB DNA-binding activity in PC-3 cells transfected with pLNCX-Myr-Akt or pLNCX. We observed an increase in NF-kappaB DNA-binding activity in PC-3 cells transfected with pLNCX-Myr-Akt (Figure 9). We also found that DIM abrogated the activation of NF-kappaB DNA-binding activity caused by pLNCX-Myr-Akt transfection (Figure 9). Collectively, these results provide evidence for a potential cross-talk between Akt and NF-kappaB pathways during DIM induced cell growth inhibition and apoptosis in prostate cancer cells.

5. DISCUSSION

DIM, the major *in vivo* product of dietary I3C, has been shown to inhibit cell growth and induce apoptosis in breast, cervical and prostate cancer cells (5-7, 11), suggesting its chemopreventive and/or therapeutic effects on cancer cells. However, the precise molecular mechanisms by which DIM inhibits cell growth and induces apoptosis have not been fully elucidated. Additionally, the effect of DIM on non-tumorigenic epithelial cells remains unknown. Here, we demonstrated that DIM significantly and selectively inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells, while CRL-2221 non-tumorigenic cells showed much less response to DIM. These results provide evidence for selective effects of DIM on cell growth and apoptosis in cancer cells. To discover the molecular mechanisms responsible for the induction of apoptosis by DIM, we investigated the effects of DIM on Akt and NF-kappaB pathways, which have been known to play important roles in cell survival and apoptotic cell death processes.

It has been known that Akt signaling pathway can be activated by various growth and survival factors such as EGF, PDGF, insulin, etc, through activation of PI3K (16, 17). PI3K activation leads to the production of phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P₃), which interacts with Akt PH domain. This interaction subsequently causes conformational changes in Akt, resulting in the exposure of two main phosphorylation sites in Akt. Akt is then activated by phosphorylation at Thr308 by Phosphoinositide-dependent protein kinase 1 (PDK1) or at Ser473 by PDK2. Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets including Bad, Forkhead transcription factors, and caspase-9, all of which are involved in apoptotic pathway (26, 27). In the apoptotic

Regulation of Akt & NF-kappaB pathways by DIM

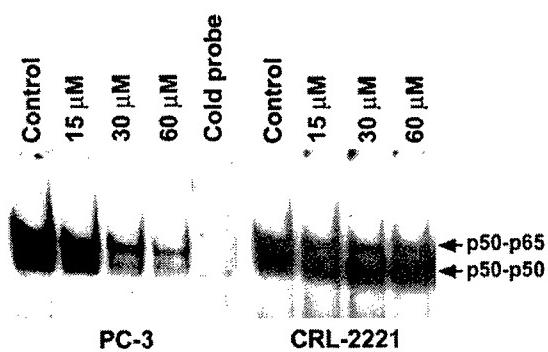


Figure 7. NF-kappaB DNA-binding activity in DIM treated PC-3 and CRL-2221 cells tested by EMSA. (Cold probe: Unlabeled NF-kappaB oligonucleotide was used as specific competitor in DNA-binding reaction).

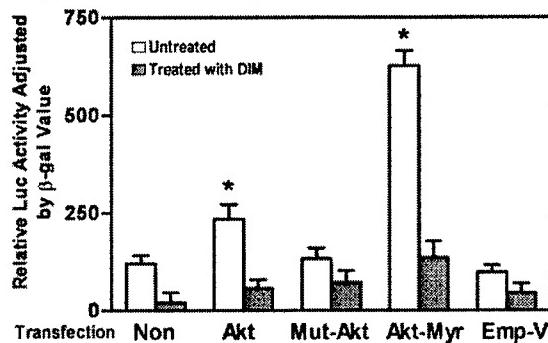


Figure 8. Luciferase activity in transfected PC-3 cells with or without DIM treatments (Non: no transfection; Akt: transfected with pLNCX-Akt; Mut-Akt: transfected with pLNCX-Akt-K179M; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

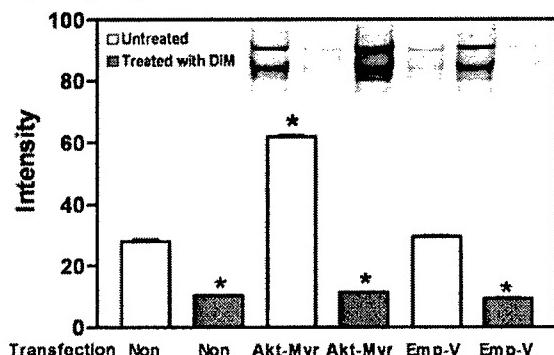


Figure 9. EMSA and densitometric analysis of NF-kappaB DNA-binding activity in transfected PC-3 cells with or without DIM treatments (Non: no transfection; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

process, dephosphorylated Bad (activated form of Bad) translocates to mitochondria, where it heterodimerizes via its BH3 domain with anti-apoptotic BCL family members such as Bcl-2 and Bcl_{XL}, promoting the onset of apoptosis (28-31). In this study, we found that DIM treatment caused the down-regulation of EGFR, suggesting that DIM could

inhibit the activation of PI3K through decrease in growth factor binding. By PI3K kinase assay, we found that DIM inactivated PI3K and abrogated the activation of PI3K caused by EGF, suggesting that DIM could inhibit Akt activity by inactivation of PI3K. Subsequently, we found no changes in the expression of un-phosphorylated Akt in DIM treated PC-3 cells. However, DIM decreased the level of phosphorylated Akt, which is the activated form of Akt. This could subsequently increase the activated Bad binding to Bcl-2 and Bcl_{XL}. We found that DIM inhibited the expression of Bcl_{XL}, suggesting an increase in the ratio of Bad/Bcl_{XL}, which could promote cancer cell to apoptotic cell death. Indeed, we observed significant induction of apoptosis in DIM treated PC-3 prostate cancer cells, and lesser apoptosis in Akt transfected PC-3 cells compared to parental PC-3 cells. These results suggest that the inhibition of PI3K/Akt signaling pathway by DIM is one of the mechanisms by which DIM induces apoptosis in prostate cancer cells.

It has been well known that NF-kappaB plays an important role in the apoptotic process (18, 20). Thus, DIM may induce apoptosis by modulating multiple components in the Akt and NF-kappaB pathways. In this study, we found that DIM selectively inhibited NF-kappaB DNA-binding activity in PC-3 prostate cancer cells. The inactivation of NF-kappaB DNA-binding activity may be another mechanism by which DIM induces apoptosis in PC-3 cells. It has been reported that the activity of NF-kappaB may be regulated by a variety of factors including Akt (22, 24). Akt has been shown to enhance the degradation of the IkappaB and induce NF-kappaB activation (32). The ability of Akt to regulate NF-kappaB activity may be through direct interaction with the IKK, supported by the observation that Akt is associated with the IKK complex *in vivo* (33). It has been demonstrated that Akt can phosphorylate and activate IKK at a critical regulatory site, Thr23, and subsequently activate NF-kappaB (24). In this study, we transfected Akt to PC-3 prostate cancer cells and tested the effect of Akt on NF-kappaB DNA-binding activity by EMSA and luciferase assay. Our results showed that Akt regulated NF-kappaB activation, and this observation is in accordance with our published data (25). Importantly, we found that DIM abrogated the NF-kappaB activation stimulated by Akt transfection, suggesting that the inhibition of NF-kappaB activity by DIM is partly mediated through Akt signaling pathway. Hence, the inactivation of DNA-binding activity of NF-kappaB by DIM appears to be responsible for DIM-induced apoptosis in PC-3 prostate cancer cells.

NF-kappaB has been described as a major culprit in cancer (34), and Akt has been known as a key molecule in cell survival (16, 17). Because of their importance in the control of cell survival and apoptotic cell death, both Akt and NF-kappaB have been believed to be very attractive therapeutic targets for cancer therapy (33, 35-37). Therefore, our results indicate that the inhibition of Akt and NF-kappaB activity could be easily achievable by DIM treatment, which inhibits cell growth and induces apoptosis in PC-3 prostate cancer cells, suggesting that DIM may be a useful agent for the prevention and/or treatment of

prostate cancer. More importantly, we found that DIM had no significant effects on cell growth, apoptosis, Akt and NF-kappaB activity in non-tumorigenic prostate epithelial cells, suggesting cancer cell specific effects of DIM. Similar cancer cell specific effects of I3C were also reported previously by our laboratory in breast epithelial cells (38). The fact that DIM selectively inhibits cell growth, Akt and NF-kappaB activation, and induces apoptosis in PC-3 prostate cancer cells, makes it a potent chemopreventive and/or therapeutic agent against prostate cancer. Our results warrant further animal and human investigations in order to fully appreciate the value of DIM in human health.

6. ACKNOWLEDGEMENT

This work was partly funded by grants from the Department of Defense (DOD Prostate Cancer Research Program DAMD17-03-1-0042 awarded to FHS) and the National Cancer Institute, NIH (1R01CA108535-01 awarded to FHS) and an AICR post-doctoral fellowship (AICR # 0013103 awarded to SRC).

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Key Words: DIM, Akt, NF-kappaB, Apoptosis, Prostate, Cancer

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Abstract Number: 466
Presentation Title: Down regulation of androgen receptor (AR) and its target gene (PSA) expressions by B-DIM leads to sensitization of prostate cancer cells to Taxotere-induced apoptosis.
Presentation Start/End Time: Sunday, Apr 17, 2005, 8:00 AM -12:00 PM
Board Number: Board #23
Author Block: *Fazlul H. Sarkar, M Mahbubur Bhuiyan, Michael Pilder, Yiwei Li.* Wayne State Univ. School of Med., Detroit, MI

Despite initial response to androgen deprivation, patients with prostate cancer usually develop hormone refractory metastasis and eventually succumb to the disease. Therefore, new therapeutic strategies are urgently needed to overcome drug resistance for the treatment of prostate cancer. Epidemiological studies have shown that the consumption of cruciferous vegetables is associated with reduced cancer risk. 3,3'-diindolylmethane (DIM) is an acid condensation product of I3C, which is present in almost all members of the cruciferous vegetable family. DIM has been shown to down-regulate AR, suggesting the growth inhibitory effects of DIM on hormone related cancers. A formulated DIM (B-DIM, BioResponse) has been shown to exhibit approximately 50% higher bioavailability. However, no molecular studies have been reported to date to elucidate the effect of B-DIM on prostate cancer cells. To investigate the effects of B-DIM on prostate cancer cells, we treated hormone sensitive LNCaP cells and hormone insensitive PC-3 cells with 0 to 60 μ M of B-DIM for one to three days. MTT, ELISA, and Western blot analysis were conducted to test cell growth inhibition, apoptosis, and the expression of AR and prostate specific antigen (PSA). Microarray analysis was also conducted to assess global alterations in gene expression profile. We found that B-DIM inhibited the growth of prostate cancer cells and induced apoptosis in a dose and time dependent manner. Microarray gene expression analysis showed that B-DIM regulated the expression of genes which are critical for the control of cell growth and apoptosis. Western blot analysis showed that B-DIM significantly inhibited AR and PSA protein expression in LNCaP prostate cancer cells. It has been recently reported that increases in androgen receptor mRNA and proteins are both necessary and sufficient to convert prostate cancer from a hormone-sensitive to a hormone refractory stage, suggesting that over-expression of AR is an important factor for acquired resistance of prostate cancer to hormone ablation therapy. Since our results showed decreased AR and PSA expression with concomitant inhibition of cell growth and induction of apoptosis by B-DIM, we suggest that the resistance acquired by prostate cancer cells to androgen ablation therapy could be reversed by B-DIM treatment. Moreover, we tested our hypothesis whether B-DIM could sensitize hormone insensitive PC-3 prostate cancer cells to Taxotere. We found that Taxotere (1 nM) in combination with 50 μ M B-DIM exerted significantly more growth inhibitory effects compared to Taxotere or B-DIM treatment alone. In conclusion, our results provide a solid foundation for devising novel therapeutic strategies for the treatment of hormone or chemo-resistant metastatic prostate cancer by including B-DIM, a non-toxic agent, with other therapeutic agents.

**96th Annual Meeting
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Citation format: Proc Amer Assoc Cancer Res 2005;46:[Abstract #].

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International Research Conference on Food, Nutrition, and Cancer

Indole-3-Carbinol and Prostate Cancer^{1,2}

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ABSTRACT Epidemiological and dietary studies have revealed an association between high dietary intake of cruciferous vegetables and decreased prostate cancer risk. Our studies have shown that indole-3-carbinol (I3C), a common phytochemical in cruciferous vegetables, and its in vivo dimeric product 3,3'-diindolylmethane (DIM) upregulate the expression of phase I and phase II enzymes, suggesting increased capacity for detoxification and inhibition of carcinogens. Studies from our laboratory and others have found that I3C can induce G1 cell-cycle arrest and apoptosis in prostate cancer cells. In addition, we found, by microarray gene expression profiling, that I3C and DIM regulate many genes that are important for the control of cell cycle, cell proliferation, signal transduction, and other cellular processes, suggesting the pleiotropic effects of I3C and DIM on prostate cancer cells. We recently found that I3C functions as an inhibitor of Akt and nuclear factor κ B (NF- κ B), which play important roles in cell survival and which are believed to be potential targets in cancer therapy. Studies have already shown that the inactivation of Akt and NF- κ B is responsible for chemosensitization of chemoresistant cancer cells. Because there is no effective treatment strategy for hormone-dependent and, most importantly, hormone-independent and metastatic prostate cancer, our strategies to sensitize prostate cancer cells to a chemotherapeutic agent by I3C and DIM is a novel breakthrough that could be used for devising novel therapies for prostate cancer. In conclusion, the results from our laboratory and from others provide ample evidence for the benefit of I3C and DIM for the prevention and the treatment of prostate cancer. *J. Nutr.* 134: 3493S–3498S, 2004.

KEY WORDS: • I3C • prostate cancer • prevention • treatment

Prostate cancer is one of the most common cancers in men and is the second leading cause of male cancer death in the United States (1). An estimated 220,900 new cases and 28,900 deaths from prostate cancer cells occurred in 2003 (1). However, men in Asia have a much lower incidence and mortality of prostate cancer than do men in North America and Europe (2,3). The differences in the cancer incidence among ethnic groups are believed to be due to different lifestyles and environmental factors. Asians who emigrated from their native countries to the United States and adopted Western lifestyles typically experienced an increasing incidence of hormone-related cancers (4,5), suggesting that the diet in

their native countries may have a role in protecting against hormone-related cancers. It has been estimated that more than two-thirds of human cancers can be prevented by modification of lifestyle, including dietary modification. The consumption of fruits, soybeans, and vegetables has been associated with reduced risk of several types of cancers (6–8). Epidemiological and dietary studies have shown an association between high dietary intake of vegetables and decreased prostate cancer risk (7,8). Indole-3-carbinol (I3C),⁴ a phytochemical common in cruciferous vegetables, inhibits carcinogenesis in animal experiments and growth of various cancer cells in culture (9–11). I3C and its in vivo dimeric product 3,3'-diindolylmethane (DIM) have received much attention in recent years as cancer preventive agents.

I3C is produced from naturally occurring glucosinolates contained in a wide variety of plants, including members of the family Cruciferae and particularly members of the genus *Brassica*. I3C is biologically active, and it is easily converted in

¹ Published in a supplement to *The Journal of Nutrition*. Presented as part of the International Research Conference on Food, Nutrition, and Cancer held in Washington, DC, July 15–16, 2004. This conference was organized by the American Institute for Cancer Research and the World Cancer Research Fund International and sponsored by BASF Aktiengesellschaft; Campbell Soup Company; The Cranberry Institute; Danisco USA Inc.; DSM Nutritional Products, Inc.; Hill's Pet Nutrition, Inc.; Kellogg Company; National Fisheries Institute; The Solae Company; and United Soybean Board. An educational grant was provided by The Mushroom Council. Guest editors for this symposium were Helen A. Norman, Vay Liang W. Go, and Ritva R. Butrum.

² This work was partly funded by grants from the Department of Defense (DOD Prostate Cancer Research Program DAMD17-03-1-0042 awarded to F.H.S.), NIH (1R01CA108535-01 awarded to F.H.S.), and Puschelberg Foundation (F.H.S.).

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⁴ Abbreviations used: ATF, activating transcription factor; CBFB, core binding factor beta; CDK, cyclin-dependent kinases; CDKI, CDK inhibitor; DIM, 3,3'-diindolylmethane; EGF, epidermal growth factor; I3C, indole-3-carbinol; IKK, I κ B kinase; MIG, mitogen inducible gene; MAPK, mitogen-activated protein kinase; NF- κ B, Nuclear factor κ B; NF-YC, nuclear factor YC; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol-3 kinase; PDK1, phosphoinositide-dependent protein kinase 1; ST16, suppressor of tumorigenicity 16; TFDP, transcription factor Dp.

vivo to DIM, which is also biologically active (12). Vegetables of the genus *Brassica* contribute most to our intake of glucosinolates and include all kinds of cabbages, broccoli, cauliflower, and Brussels sprouts. All glucosinolates share a common basic skeleton containing a glucose group, a side chain, and a sulfonated oxime moiety but differ in the side chain. Glucosinolates with an indole side chain form indoles. The most prevalent glucosinolate with an indole side chain is glucobrassicin, which is predominant in brassica vegetables. When hydrolysis occurs, glucobrassicin forms an unstable isothiocyanate that degrades to I3C (13). Under the acidic conditions of the stomach, I3C undergoes extensive and rapid self-condensation reactions to form DIM (13,14) (Fig. 1).

Because of its anticarcinogenic effects in experimental animals (11,15,16) and humans (17–19), I3C has received special attention as a possible chemopreventive agent (13). I3C has also been found to inhibit the growth of various cancer cells (20–23) and possibly to inhibit breast cancer invasion and migration (24,25). Because of its pleiotropic effects, studies on I3C as a cancer chemopreventive agent have increased significantly in recent years. However, the molecular mechanisms by which I3C exerts its tumor suppressive effects on prostate cancers have not been fully elucidated. In this article, we summarized our data regarding the inhibitory effects of I3C and DIM on prostate cancer cells and provided a comprehensive view on the molecular mechanisms of cancer prevention and treatment by I3C and DIM.

I3C inhibits cell growth and induces cell-cycle arrest in prostate cancer cells

I3C inhibits the growth of breast, prostate, colon, and cervical cancer cells (9,20,26–28). Data from our laboratory showed that I3C and DIM inhibited the growth of PC3 prostate cancer cells (29–31). The inhibition of cell growth was

found to be dose and time dependent. I3C at 60 $\mu\text{mol/L}$ and DIM at 30 $\mu\text{mol/L}$ significantly inhibited PC3 cell growth. This growth inhibition could be due to cell-cycle arrest, which ultimately results in the cessation of cell proliferation. By flow cytometry analysis, we found that I3C induced G1 cell-cycle arrest in PC3 prostate cancer cells (29), in accord with the report showing that I3C induces G1 cell-cycle arrest in breast cancer cells (32).

Because the induction of cell-cycle arrest in prostate cancer cells by I3C could be mediated via regulation of the expression of genes involved in the control of the cell cycle, we further examined the status of cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors (CDKI) in I3C-treated PC3 prostate cancer cells. By Western blot analysis, we found that I3C downregulated the expression of CDK6 and upregulated the expression of p21^{WAF1} and p27^{KIP1} in a dose-dependent manner (29). We immunoprecipitated cyclin D1 and cyclin E complexes, and detected CDK6, p21^{WAF1}, and p27^{KIP1} in the complexes. The results showed that I3C decreased CDK6 binding to cyclin D1 and increased p21^{WAF1} and p27^{KIP1} binding to cyclin D1 and cyclin E complexes (29), suggesting the inhibition of cell-cycle progression. We also found that I3C inhibited the CDK6 kinase activity (29), which plays important roles in G1 regulation. These findings were consistent with results showing cell-growth inhibition and cell-cycle arrest induced by I3C, suggesting that I3C inhibits the growth of prostate cancer cells through regulation of genes related to the control of cell proliferation and the cell cycle. Reports from other laboratories also showed that I3C inhibited the expression of CDK6 and induced G1 arrest in breast cancer cells (32,33). The upregulation of p21^{WAF1} and p27^{KIP1} and the downregulation of CDK6 may be one of the molecular mechanisms by which I3C inhibits prostate cancer cell growth and induces cell-cycle arrest (Fig. 2).

I3C induces apoptosis in prostate cancer cells

I3C induces apoptotic cell death in breast cancer cells (27,34). We conducted the DNA ladder assay, the poly(ADP-ribose) polymerase (PARP) assay, and the flow cytometric analysis with 7-amino actinomycin D (7AAD) staining to detect apoptosis in PC3 prostate cancer cells treated with I3C. By using these different techniques, we found that I3C at 60–100 $\mu\text{mol/L}$ induced apoptosis in PC3 prostate cancer cells (29). DNA ladder formation and PARP cleavage were observed in prostate cancer cells treated with I3C for 48 h. Flow cytometry analysis revealed that the number of apoptotic cells increased up to 58.49% with I3C treatment at 100 $\mu\text{mol/L}$ for 48 h and reached up to 80% with longer I3C treatment (30). These results clearly demonstrated that I3C induces apoptosis in prostate cancer cells, which is consistent with studies in breast cancer cells (27,34).

To find the molecular mechanisms by which I3C induces apoptosis, we investigated the alteration of protein expression of genes related to the apoptotic pathway. Bax, Bcl-2, and Bcl_{XL} play important roles in determining whether cells will undergo apoptosis (35,36). Bax promotes apoptotic cell death, whereas Bcl-2 and Bcl_{XL} protect cells from apoptosis. The ratio of Bax to Bcl-2 rather than Bcl-2 alone was reported to be an important factor for determining whether cells survive or undergo apoptosis (37). By Western blot analysis, we found decreases in Bcl-2 and Bcl_{XL} protein expression in PC3 prostate cancer cells treated with I3C at 60 $\mu\text{mol/L}$ for 48 h and longer (29,30). However, the expression of Bax was upregulated after I3C treatment for 24 h. The ratio of Bax to Bcl-2 was significantly increased after 24 h of I3C treatment corre-

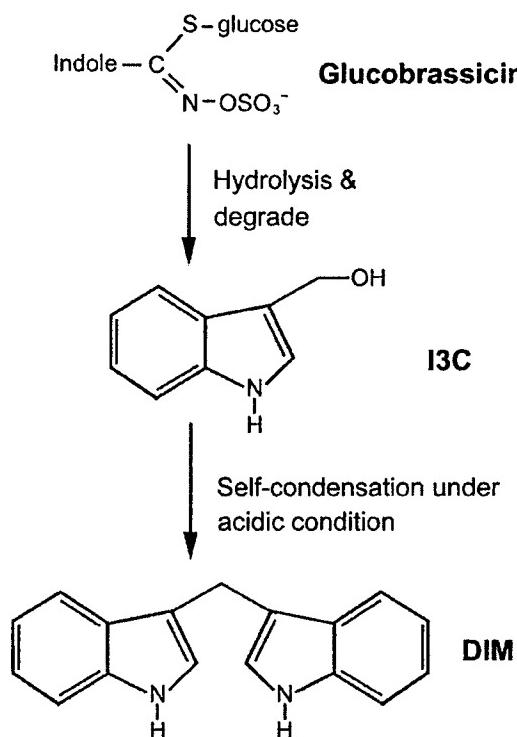


FIGURE 1 Molecular structure and metabolism of I3C and DIM.

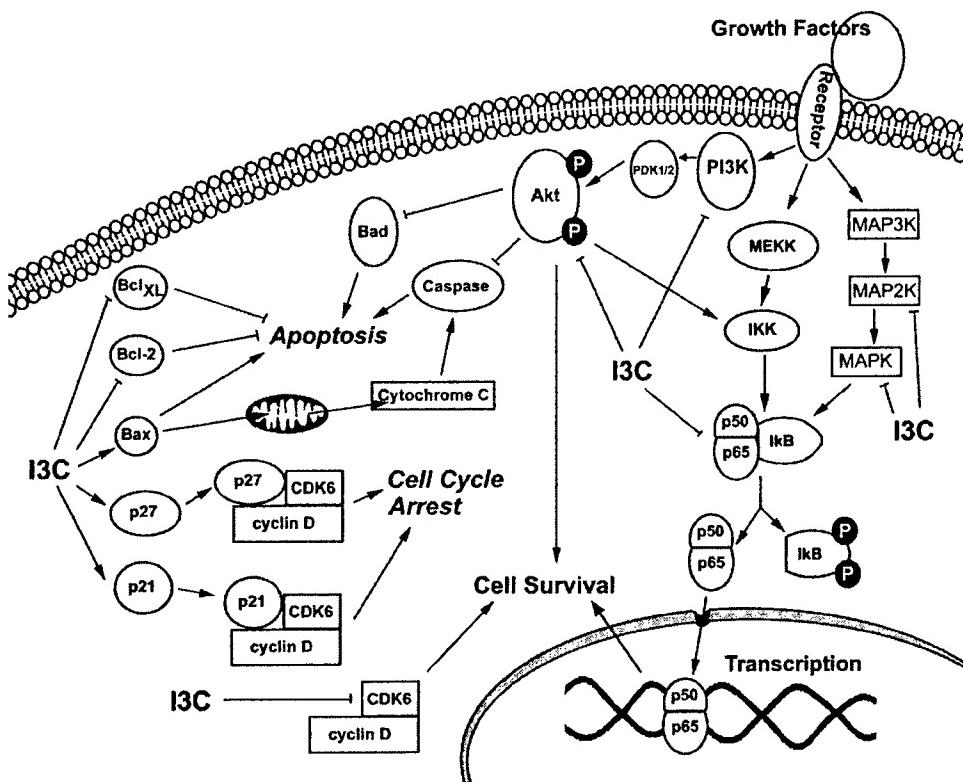


FIGURE 2 The effects of I3C on cell cycle, apoptosis, NF- κ B, Akt, and MAPK pathways.

sponding to a significant increase in apoptotic cells after I3C treatment for 48 h. Similar results were also observed in I3C-treated breast cancer cells in our laboratory (38,39). These results suggest that upregulation of Bax and downregulation of Bcl-2 and Bcl_{XL} may be one molecular mechanism by which I3C induces apoptosis (Fig. 2).

Bax translocation from cytosol into mitochondria also plays important roles in the induction of apoptosis (40). The translocation of Bax from cytosol into mitochondria targets the mitochondrial intermembrane contact sites, causing the mitochondrial permeability transition, loss of mitochondrial potential, release of cytochrome c, subsequent activation of caspases, and DNA fragmentation, resulting in apoptosis (40,41). To further explore the mechanism of I3C-induced apoptosis, we investigated Bax localization, mitochondrial potential, and cytochrome c in both MCF10A nontumorigenic cells and MCF10CA1a cancer cells treated with I3C (42). By using immunostaining and confocal imaging techniques, we observed the translocation of Bax from the cytosol into the mitochondria in both cell lines treated with I3C. However, the loss of mitochondrial potential and the release of cytochrome c induced by I3C were only observed in MCF10CA1a breast cancer cells. No such effects or significant apoptosis were observed in MCF10A nontumorigenic cells, suggesting that I3C-induced loss of mitochondrial potential is a more important event for the release of cytochrome c and induction of apoptosis in cancer cells. We also observed that DIM selectively induces apoptosis in PC3 prostate cancer cells but not in CRL-2221 nontumorigenic cells, suggesting that I3C and DIM may be the ideal agents for the prevention and the treatment of prostate and other cancers.

I3C inhibits the nuclear factor κ B pathway in prostate cancer cells

The nuclear factor κ B (NF- κ B) pathway plays an important role in many physiological processes in cellular signaling (43–

45). In human cells, NF- κ B is sequestered in the cytoplasm through tight association with its inhibitory protein, IkB. The activation of NF- κ B occurs through site-specific phosphorylation of IkB by IkB kinase (IKK). IkB is subsequently degraded by the 26S proteasome. NF- κ B becomes free from IkB and translocates into the nucleus for binding to NF- κ B-specific DNA-binding sites, regulating target gene transcription. IKK- α also phosphorylates histone H3 and regulates the activation of NF- κ B-directed gene expression. NF- κ B can be activated by many types of stimulators (43–46), including carcinogens [TNF, 9,10-dimethyl-1,2,benzanthracene (DMBA), cigarette smoke condensate, etc.], tumor promoters (phorbol myristate acetate, etc.), stress (pH, hypoxia, heavy metals, hydrogen peroxide, etc.), endotoxin (LPS, etc.), apoptosis inducers (chemotherapeutic agents, cytokines, etc.), infection (bacterial, viral, etc.), and cytokines [IL-1, IL-17, IL-18, epidermal growth factor (EGF), etc.]. Activated NF- κ B controls the expression of genes that are involved in controlling cell proliferation, differentiation, apoptosis, inflammation, stress response, angiogenesis, tumor promotion and metastasis, and other cellular and physiological processes (43–45). Because of its critical effects on tumor development and progression, NF- κ B has been described as a major culprit and a therapeutic target in cancer (43,46,47). Inhibition of NF- κ B activation is generally believed to suppress tumorigenesis and the progression of tumors.

We used an electrophoretic mobility shift assay to investigate whether I3C treatment inhibits NF- κ B DNA binding activity in PC3 prostate cancer cells (29). PC3 cells were treated with I3C at 60 μ mol/L for 48 h or TNF- α at 50 μ g/L for 15 min. Nuclear extracts were harvested from samples, incubated in binding buffer with ³²P-labeled NF- κ B consensus oligonucleotide, and subjected to 8% nondenatured polyacrylamide gel. Autoradiography of the dried gel showed that TNF- α treatment stimulated NF- κ B activation as expected; however, I3C at 60 μ mol/L significantly inhibited NF- κ B

DNA binding, corresponding to inhibition of cell proliferation and induction of apoptosis by I3C in PC3 prostate cancer cells. These results suggest that inhibition of NF- κ B pathway by I3C may be another molecular mechanism by which I3C inhibits cell proliferation and induces apoptosis (Fig. 2).

I3C inhibits Akt pathway in prostate cancer cells

The Akt signaling pathway is another important signal transduction pathway in human cells and plays a critical role in controlling the balance between cell survival and apoptosis (48–50). This pathway can be activated by various growth and survival factors, such as EGF, platelet-derived growth factor, insulin, etc., through activation of phosphatidylinositol-3 kinase (PI3K) (48). PI3K activation leads to the production of phosphatidylinositol-3,4,5-trisphosphate, which interacts with the Akt PH domain. The interaction subsequently causes Akt conformational changes, resulting in exposure of 2 main phosphorylation sites in Akt. Akt is then activated by phosphorylation at Thr308 by phosphoinositide-dependent protein kinase 1 (PDK1) or at Ser473 by PDK2 (48). Activated Akt promotes cell survival by inhibiting apoptosis through its ability to phosphorylate and to inactivate several targets, including Bad, Forkhead transcription factors, and caspase-9 (50), all of which are involved in the apoptotic pathway. More importantly, Akt also activates the NF- κ B pathway through phosphorylation of molecules in the NF- κ B pathway (51), suggesting its role in promoting cell survival. Because of its importance in cell survival, Akt is also believed to be a target in cancer therapy.

To investigate whether I3C-induced cell-growth inhibition and induction of apoptosis occurs through the Akt pathway, we examined Akt status in PC3 cells treated with I3C at 30–100 μ mol/L by Western blot analysis (30). We did not find any change in the level of total Akt protein expression after I3C treatment. However, we observed a decrease in the phosphorylated Akt protein at Ser473 and Thr308 in the I3C-treated PC3 cells compared with control cells, suggesting inactivation of Akt kinase after I3C treatment. Immunoprecipitation and Akt kinase assay showed that I3C downregulated Akt kinase activity in PC3 cells, consistent with the data from Western blot analysis. We also examined the Akt status in the PC3 cells pretreated with I3C followed by EGF stimulation. We found that EGF upregulated Akt kinase activity; however, I3C pretreatment abrogated EGF-induced activation of Akt. These data clearly demonstrated that I3C inhibits Akt activation both with and without stimulation. From the gene expression profiles of I3C-treated PC3 cells, we also found downregulation of PI3K expression (31), corresponding to our results, showing I3C-induced inactivation of Akt kinase activity. Inhibition of Akt activity with downregulation of Bcl-2 and Bcl_{XL} may result in the inhibition of survival signals and may also induce apoptotic signals. Thus, the inhibition of Akt pathways by I3C may be another molecular mechanism by which I3C induces apoptosis in prostate cancer cells (Fig. 2).

We also showed that forced overexpression of Akt in prostate cancer cells by Akt gene transfection leads to the activation of NF- κ B (52). The activation of Akt and NF- κ B is believed to be responsible for the resistance to chemotherapeutic agents, which is the major cause for treatment failure in cancer chemotherapy. Inhibition of NF- κ B or Akt can potentiate the anticancer effect of chemotherapeutic agents (53,54). We found that I3C inhibited the activation of Akt and NF- κ B and induced apoptosis, suggesting that I3C can sensitize cancer cells to apoptosis induced by chemotherapeutic agents. By cell growth inhibition assay, we found that combination treat-

ment with I3C and cisplatin significantly inhibited the growth of PC-3 prostate cancer cells compared with monotherapy (Fig. 3). These results suggest that I3C and DIM can potentiate the anticancer effect of chemotherapeutic agents through inactivation of Akt and NF- κ B.

I3C and DIM regulate gene expression profiles in prostate cancer cells

To explore the precise molecular mechanisms by which I3C and DIM exert their pleiotropic effects on prostate cancer cells, we used the high-throughput gene chip, which contains 22,215 known genes, to determine the alteration of gene expression profiles of PC3 prostate cancer cells exposed to I3C or DIM (31). By microarray analysis, we found that both I3C and DIM regulated many genes related to cell proliferation, cell-cycle control, apoptosis, signal transduction, oncogenesis,

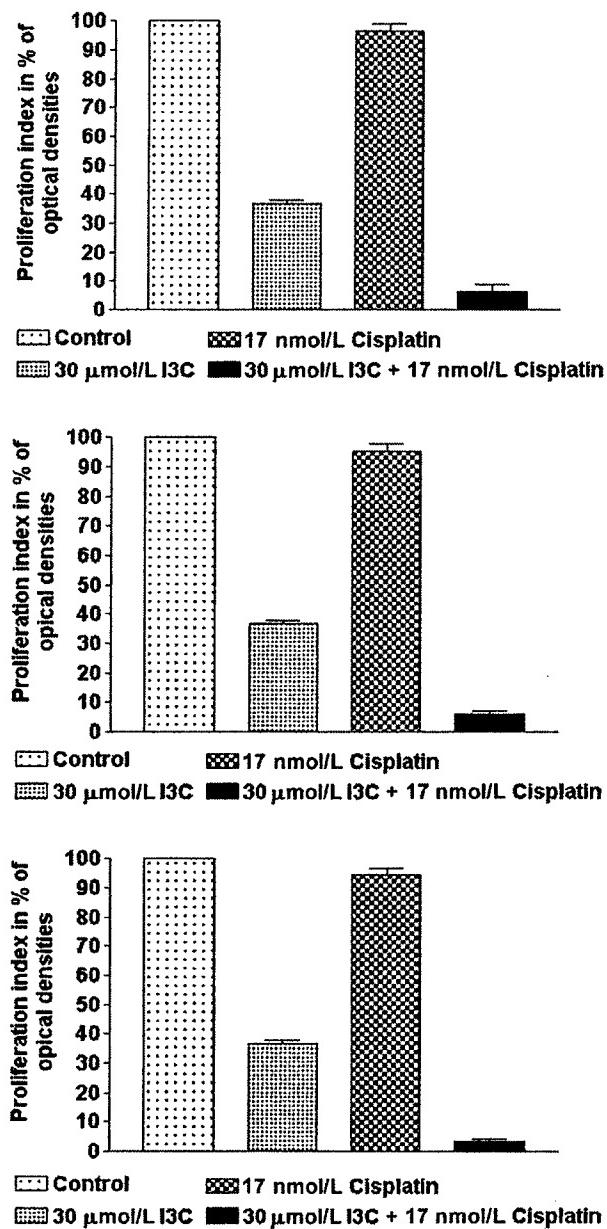


FIGURE 3 Growth inhibition of PC-3 prostate cancer cells exposed to I3C and cisplatin for 72 h (repeated 3 times).

and transcription regulation, suggesting that I3C and DIM alter biological processes and molecular functions in PC3 cells through a variety of cell-signaling pathways. The global gene expression profiles of prostate cancer cells after I3C or DIM treatment provided important information for further investigation of the molecular mechanisms by which I3C and DIM inhibit prostate cancer cells.

EGF receptor, transforming growth factor- β , and fibroblast growth factor play important roles in promoting cell growth and angiogenesis. From microarray analysis, we found that I3C and DIM downregulated their expression, corresponding to the growth inhibitory effects of I3C and DIM. Cyclin E, Bcl-2, activating transcription factor (ATF), and mitogen inducible gene (MIG) promote cell-cycle progression and inhibit apoptosis (55,56). We found that I3C and DIM inhibited the expression of cyclin E2, ATF5, MIG-2, and Bcl-2, and induced the expression of p57^{KIP2}, suggesting the effects of I3C and DIM on the induction of cell-cycle arrest and apoptosis.

From microarray analysis, we also found that I3C and DIM also regulated other cell-signal transduction pathways, e.g., the mitogen-activated protein kinase (MAPK) pathway, which consists of a 3-tiered kinase core where a MAP3K activates a MAP2K that activates a MAPK (57,58). Because it promotes the activation of NF- κ B and cell survival, MAPK has also been believed to be a target for cancer prevention and therapy (58). We observed a decrease in the expression of MAP2K3, MAP2K4, MAP4K3, and MAPK3 after I3C and DIM treatment, suggesting the inhibitory effects of I3C and DIM on the MAPK pathway, which could result in the inhibition of cancer-cell survival (Fig. 2).

Pol II transcription factors [transcription factor Dp (TFDP), nuclear factor YC (NF-YC), etc.] play important roles in transcription, cell-cycle progression, and oncogenesis (59,60). In addition, core binding factor beta (CBFB) and suppressor of tumorigenicity 16 (ST16) are also involved in the processes of oncogenesis. CBFB forms fusion proteins with other gene products and promotes oncogenesis, whereas ST16 suppresses the oncogenesis (59,60). Our results from gene expression profiles showed that I3C and DIM downregulated the expression of TFDP1, NF-YC, and CBFB, and upregulated ST16 expression, suggesting that I3C and DIM can inhibit transcription and oncogenesis in PC3 prostate cancer cells.

Summary and perspective

In conclusion, I3C and DIM from the natural foods of the family Cruciferae exert anticancer effects mediated through the regulation of the cell cycle, cell proliferation, apoptosis, oncogenesis, transcription, and cell-signal transduction. The inactivation of Akt, NF- κ B, MAPK, and Bcl-2 signaling pathways may be the molecular mechanisms by which I3C and DIM inhibit cell growth and induce apoptosis in prostate cancer cells. Because I3C functions as an inhibitor of NF- κ B and Akt activation and induces apoptosis, I3C and DIM may also sensitize prostate cancer cells to apoptosis induced by chemotherapeutic agents. However, further *in vitro* and *in vivo* investigations, along with clinical trials, are needed to find whether I3C and DIM can fulfill their promise as chemopreventive agents, therapeutic agents, or both against human prostate cancer.

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Review



Fundamental and Molecular
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Cell signaling pathways altered by natural chemopreventive agents

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Received 19 January 2004; received in revised form 26 April 2004; accepted 27 April 2004

Abstract

Epidemiological studies have indicated a significant difference in the incidence of cancers among ethnic groups, who have different lifestyles and have been exposed to different environmental factors. It has been estimated that more than two-thirds of human cancers, which are contributed by mutations in multiple genes, could be prevented by modification of lifestyle including dietary modification. The consumption of fruits, soybean and vegetables has been associated with reduced risk of several types of cancers. The *in vitro* and *in vivo* studies have demonstrated that some dietary components such as isoflavones, indole-3-carbinol (I3C), 3,3'-diindolylmethane (DIM), curcumin, (−)-epigallocatechin-3-gallate (EGCG), apigenin, etc., have shown inhibitory effects on human and animal cancers, suggesting that they may serve as chemopreventive agents. Experimental studies have also revealed that these components regulate the molecules in the cell signal transduction pathways including NF- κ B, Akt, MAPK, p53, AR, and ER pathways. By modulating cell signaling pathways, these components, among other mechanisms, activate cell death signals and induce apoptosis in precancerous or cancer cells, resulting in the inhibition of cancer development and/or progression. This article reviews current studies regarding the effects of natural chemopreventive agents on cancer-related cell signaling pathways and provides comprehensive knowledge of the biological and molecular roles of chemopreventive agents in cancer cells.

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Keywords: Signaling pathway; Cancer; Prevention

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1. Introduction

It has been known that most human cancers are induced by environmental factors including chemical, radioactive and biological factors that exist in our living environment. There are significant differences in the cancer incidence and mortality among ethnic groups, who have different lifestyles and have been exposed to different environmental factors [1]. It has been estimated that more than two-thirds of human cancers could be prevented by modification of lifestyle including dietary modification [2]. The consumption of fruits, soybean and vegetables has been associated with reduced risk of several types of cancers [3–5]. The experimental *in vitro* and *in vivo* studies have demonstrated that some dietary components such as isoflavones, indole-3-carbinol (I3C), 3,3'-diindolylmethane (DIM), curcumin, (−)-epigallocatechin-3-gallate (EGCG), apigenin, have inhibitory effects on human cancers [6–13] suggesting that they may serve as chemopreventive agents.

The dietary components as chemopreventive agents have received much attention among the public and the medical community. Soy isoflavones including genistein, daidzein, and glycinein, mainly derived from soybean have been found to inhibit cancer growth *in vivo* and *in vitro* [14–16]. I3C and its *in vivo* dimeric product DIM, produced from naturally occurring glucosinolates in the family Cruciferae, have shown the inhibition of cancer cell growth through the modulation of genes that are related to the control of cell proliferation, cell cycle, apoptosis, signal transduction, oncogenesis, and transcription regulation [8,9]. Curcumin, a natural compound present in turmeric and possessing both anti-inflammatory and antioxidant effects, has

been studied as a chemopreventive agent in several cancer models [11,17]. Beneficial effects attributed to green tea, such as its anticancer and antioxidant properties, are believed to be mainly mediated by EGCG [4,18]. Apigenin, one of the flavonoids, is widely distributed in many fruits and vegetables, and has been shown to possess anti-inflammatory and anticancer effects [19,20]. It is becoming clear that these dietary components exert their pleiotropic effects on cancer cells, affecting cell survival and physiological behaviors. However, the precise molecular mechanisms of actions of these components have not been fully elucidated, although the data from published literature does indicate that these components regulate transcription, translation and activation of the molecules in the cell signal transduction pathways. Among the cell signaling pathways, NF-κB, Akt, MAPK, p53, AR, and ER pathways are more important signaling pathways related to cancer development and progression. This article reviews current studies regarding the effects of natural chemopreventive agents on these cancer-related cell signaling pathways and provides comprehensive knowledge of the biological and molecular roles of chemopreventive agents in cancer cells.

2. Effects of chemopreventive agents on NF-κB

It has been well accepted that nuclear factor-κB (NF-κB) signaling pathway plays important roles in the control of cell growth, apoptosis, inflammation, stress response, and many other physiological processes [21–25]. There are several important molecules such as NF-κB, IκB, IKK, within NF-κB signaling pathway (Fig. 1). However, NF-κB is the key protein

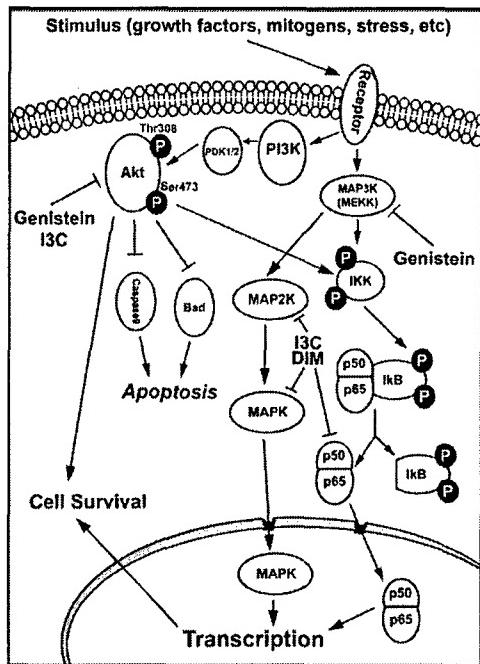


Fig. 1. Akt, NF-κB, and MAPK signaling pathways and the effects of genistein, I3C, and DIM on the pathways.

in the pathway, and has been described as a major culprit and a therapeutic target in cancer [26–29]. The data from experimental studies have demonstrated that genistein, I3C, curcumin, EGCG, and apigenin inhibit activation of NF-κB in different cancer cell lines, suggesting the inhibitory effects of these agents on cancer cells [8,20,30–33].

Our laboratory examined NF-κB DNA-binding activity in genistein treated PC3 and LNCaP prostate cancer cells by electrophoresis mobility shift assay (EMSA) [31]. The results showed that 50 μM genistein significantly inhibited the NF-κB DNA-binding activity in both cell lines. Furthermore, genistein pretreatment also abrogated the activation of NF-κB stimulated by H₂O₂ or TNF-α. Immunochemistry and confocal microscopic analysis also showed that TNF-α treatment significantly increased nuclear staining of the NF-κB p50 and p65 subunits, however, 24 h pretreatment of cells with genistein prior to TNF-α stimulation blocked p50 and p65 nuclear translocation. These results clearly demonstrate that genistein inhibits the translocation of NF-κB subunits to the nucleus, suggesting that genistein may reduce the NF-κB binding

to its target DNA and thereby inhibit the transcription of target gene. Similar results in human lung epithelial cells and myeloid cells have been reported by other investigators [34,35]. It is important to note that the concentration of genistein used in experimental study is achievable in humans. The study reported by Busby et al. has shown that up to 27.46 ± 15.38 μM of genistein in human plasma can be achieved after receiving genistein supplement at a dose of 16.0 mg/kg [36], suggesting the bioavailability of genistein from supplement.

It has been found that oxidative stress activates NF-κB DNA binding activity [37,38]. Because soy isoflavones have been known as antioxidants, their inhibitory effects on oxidative stress may be mediated through inhibition of NF-κB DNA binding activity. We investigated whether soy isoflavone supplementation could inactivate NF-κB in vivo and reduce oxidative damage in lymphocytes in human volunteers [39]. We found that when human volunteers received 50 mg of soy isoflavone supplements Novasoy™ (Archer Daniels Midland Company, Decatur, IL, USA; containing genistein, daidzein, and glycitein at a 1.3:1:0.3 ratio) twice daily for 3 weeks, TNF-α failed to activate NF-κB activity in lymphocytes harvested from these volunteers, while lymphocytes from these volunteers collected prior to soy isoflavone intervention showed activation of NF-κB DNA binding activity upon TNF-α treatment in vitro. These results demonstrated that soy isoflavone supplementation had a protective effect against TNF-α induced NF-κB activation in humans. We further measured the levels of 5-OHmU, a modified DNA base that represents endogenous status of cellular oxidative stress, in the peripheral blood lymphocytes of human volunteers before and after supplementation with Novasoy™. The mean value of 5-OHmU before supplementation was 156.7 ± 25.72 and it was decreased to 60.83 ± 12.61 ($P < 0.01$) after 3 weeks of soy supplementation. These results provide evidence showing that soy isoflavones function as antioxidants and inhibit NF-κB activation, suggesting that these effects of soy isoflavones may be responsible for its cancer chemopreventive activity.

It has been known that NF-κB could be activated by phosphorylation and degradation of IκB [40]. IκB could be phosphorylated by activated IκB kinase (IKK), and IKK could be phosphorylated and activated by mitogen activated kinase kinase 1 (MEKK1), one of the molecules in MAPK pathway [41–43]. We found

that genistein treatment reduced the amount of phosphorylated I κ B, demonstrating that genistein inhibits the phosphorylation of I κ B and ultimately prevents the translocation of NF- κ B to the nucleus. Moreover, we found that genistein treatment did not alter the protein expression of MEKK1; however, it did inhibit MEKK1 kinase activity in prostate cancer cells. These results suggested that genistein could inhibit MEKK1 kinase activity, which could be responsible for the decreased phosphorylation of I κ B and, thereby, result in the inactivation of NF- κ B (Fig. 1).

Our laboratory also investigated whether I3C treatment could modulate NF- κ B DNA binding activity in PC3 prostate cancer cells by EMSA [8]. The results showed that 60 μ M I3C significantly inhibited NF- κ B DNA binding activity with induction of apoptosis in PC3 prostate cancer cells, suggesting that inhibition of NF- κ B signaling pathway may be one of the molecular mechanisms by which I3C induces apoptosis in cancer cells (Fig. 1).

Other chemopreventive agents also show their inhibitory effects on NF- κ B pathway. It has been reported that curcumin inhibited IKK, suppressed both constitutive and inducible NF- κ B activation, and potentiated TNF-induced apoptosis [30]. Curcumin also showed strong antioxidant and anticancer properties through regulating the expression of genes that require the activation of activator protein 1 (AP1) and NF- κ B [44]. It has been reported that EGCG treatment resulted in a significant dose- and time-dependent inhibition of activation and translocation of NF- κ B to the nucleus by suppressing the degradation of I κ B α in the cytoplasm [45,46]. EGCG also showed to inhibit activation of IKK and phosphorylation of I κ B α [32,47]. It has been found that EGCG had a concurrent effect on two important transcription factors p53 (stabilization of p53) and NF- κ B (negative regulation of NF- κ B activity), causing a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis [13]. Apigenin treatment also resulted in down-modulation of the constitutive expression of NF- κ B/p65 [33].

3. Effects of chemopreventive agents on Akt

Akt plays critical roles in mammalian cell survival signaling and has been shown to be activated in various cancers [48,49]. It has been known that Akt is acti-

vated by phospholipid binding and phosphorylation at Thr308 by PDK1 or at Ser473 by PDK2 [50]. Activated Akt functions to promote cell survival by inhibiting apoptosis through inactivation of several pro-apoptotic factors including Bad, Forkhead transcription factors, and caspase-9 [51–53]. Recent studies have also shown that Akt regulates the NF- κ B pathway via phosphorylation and activation of molecules in the NF- κ B pathway [54,55] (Fig. 1). Like NF- κ B, Akt has also been believed to be an attractive target for cancer prevention or treatment [56].

The data from our laboratory showed that genistein inhibit both Akt and NF- κ B pathways [57]. We found no alteration on the total Akt protein expression in genistein treated PC3 cells; however, decreases in the phosphorylated Akt protein at Ser473 and the Akt kinase activity were observed in genistein-treated PC3 cells, suggesting the inactivation of Akt after genistein treatment. We also found that genistein pretreatment abrogated the activation of Akt by EGF. To further explore the inhibitory mechanism of genistein on Akt and NF- κ B pathways, Akt expression construct (pLNCX-Akt) was transiently co-transfected with NF- κ B-Luc reporter construct into PC3 prostate cancer cells. Luciferase assay showed an increased luciferase activity in PC3 cells co-transfected with pLNCX-Akt and NF- κ B-Luc. However, genistein inhibited the luciferase activity in PC3 cells co-transfected with pLNCX-Akt and NF- κ B-Luc, and abrogated the activation in PC3 cells co-transfected with pLNCX-Akt and NF- κ B-Luc followed by EGF stimulation. These results were further confirmed by examining NF- κ B DNA-binding activity in transfected cells using EMSA, which showed similar results to those of transfection and luciferase assay. We also observed similar results in MDA-MB-231 breast cancer cells [58]. These results suggest that genistein exerts its inhibitory effects on NF- κ B pathway through Akt pathway, and that down-regulation of NF- κ B and Akt signaling pathways by genistein may be one of the molecular mechanisms by which genistein inhibits cancer cell growth and induces apoptosis (Fig. 1).

To explore the effect of I3C on Akt pathway, we examined Akt status in PC3 cells treated with 30, 60, and 100 μ M I3C by Western blot, immunoprecipitation, and kinase assays [59]. We found a decrease in the phosphorylated Akt protein at Ser473 and Thr308 in I3C treated PC3 cells, suggesting inactivation of Akt after I3C treatment. These results were confirmed by

Akt kinase assay, which showed a decrease in the Akt kinase activity in I3C treated PC3 cells. We also found that I3C pretreatment abrogated the activation of Akt by EGF. From the gene expression profiles of PC3 cells exposed to I3C, we found down-regulation of PI3K expression, consistent with our results showing inactivation of Akt kinase by I3C [9]. These data demonstrated that I3C inhibited Akt signaling pathway, which may result in the inhibition of survival signals and the induction of apoptotic signals (Fig. 1).

Similar to our results about the effect of genistein on Akt, Kumar et al. have reported that an analogue of curcumin, 4-hydroxy-3-methoxybenzoic acid methyl ester (HMBME), targeted the Akt signaling pathway, inhibited the proliferation of human and mouse prostate cancer cells and induced apoptosis [60]. Transfection experiment showed that overexpression of constitutively active Akt reversed the HMBME-induced growth inhibition and apoptosis, demonstrating the direct role of Akt signaling in HMBME-mediated growth inhibition and apoptosis. HMBME also decreased the level of phosphorylated Akt, inhibited Akt kinase activity, and reduced DNA-binding activity of NF- κ B [60]. Several reports by other investigators also suggest that curcumin has molecular targets within the Akt signaling pathways, and the inhibition of Akt activity may facilitate inhibition of proliferation and induction of apoptosis in cancer cells [61,62].

Recent report has shown that EGCG from green tea inhibits VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule, suggesting inhibitory effect of EGCG on Akt signaling pathway [63]. Masuda et al. also found that treatment with EGCG inhibited the constitutive activation of the Akt, EGFR, and Stat3 in both YCU-H891 head and neck squamous cell carcinoma and MDA-MB-231 breast carcinoma cell lines [64].

4. Effects of chemopreventive agents on MAPK

In addition to NF- κ B and Akt pathways, MAPK has received increasing attention as a target molecule for cancer prevention and therapy. It has been reported that activation of the MAPK pathways may cause the induction of phase II detoxifying enzymes, and inhibition of MAPK pathways may inhibit AP-1-mediated gene ex-

pression [65]. MAPK pathway consists of a three-tiered kinase core where a MAP3K activates a MAP2K that activates a MAPK (ERK, JNK, and p38), resulting in the activation of NF- κ B, cell growth, and cell survival [66,67] (Fig. 1).

We have utilized the high-throughput gene chip, which contains 22,215 known genes, to determine the alternation of gene expression profiles of PC3 prostate cancer cells exposed to I3C or DIM [9]. From microarray data, we observed down-regulation in the expression of MAP2K3, MAP2K4, MAP4K3, and MAPK3 by I3C and DIM treatment, suggesting the inhibitory effects of I3C and DIM on MAPK pathway. The down-regulation of the important molecules in MAPK pathway may result in the inhibition of cancer cell survival (Fig. 1).

The ability of curcumin to modulate MAPK signaling pathway might contribute to the inhibition of inflammation by curcumin. Salh et al. reported that curcumin is able to attenuate experimental colitis through a reduction in the activity of p38 MAPK [68].

The reported effects of EGCG on MAPK pathway are controversial. EGCG showed strong inhibition of tyrosine kinase and MAPK activities in transformed NIH-pATM ras fibroblasts, without affecting the kinases in the normal cells [69]. Katiyar et al. reported that treatment of H₂O₂ resulted in phosphorylation of ERK1/2, JNK, and p38 in human epidermal keratinocytes [70]. When these cells were pretreated with EGCG, H₂O₂-induced phosphorylation of ERK1/2, JNK, and p38 was found to be significantly inhibited. These findings demonstrate that EGCG has the potential to inhibit oxidative stress-mediated phosphorylation of MAPK signaling pathways. Maeda-Yamamoto et al. also reported that EGCG inhibited the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), and suppressed p38 MAPK activity in human fibrosarcoma HT1080 cells [71]. However, EGCG has also been found to activate all three MAPKs (ERK, JNK and p38) in a dose- and time-dependent manner in human hepatoma HepG2-C8 cells [72]. In breast cancer cell line T47D, catechin (containing approximately 53% of EGCG) showed to phosphorylate JNK/SAPK and p38. The phosphorylated JNK/SAPK and p38 inhibited the phosphorylation of cdc2, and regulated the expression of cyclin A, cyclin B1, and cdk proteins, thereby causing G2 arrest [73]. It is possible that activation of MAPK by low concentration of EGCG re-

sults in induction of ARE-mediated gene expression, whereas higher concentration of EGCG causes activation of MAPKs such as JNK leading to apoptosis [72].

5. Effects of chemopreventive agents on p53

p53 is a tumor suppressor and transcription factor. It is a critical regulator in many cellular processes including cell signal transduction, cellular response to DNA-damage, genomic stability, cell cycle control, and apoptosis. As a tumor suppressor, functional p53 activates the transcription of downstream genes such as p21^{WAF1} and Bax to induce the apoptotic process, inhibiting the growth of DNA damaged cells or cancer cells [74–76]. The status of p53 is thought to be an important mediator in the cellular response to chemotherapy [77].

In order to investigate whether genistein inhibits proliferation and induces apoptosis through p53 pathway in non-small cell lung cancer (NSCLC) cells, we measured cell growth inhibition, apoptosis, and gene expression related to apoptosis in genistein treated H460 cells, which harbor wild type p53, and H322 cells that possess a mutation in the p53 gene (codon 248, CGG to CTG, Arg to Leu) [78,79]. Genistein was found to inhibit both H460 and H322 cell growth in a dose-dependent manner. DAPI staining, poly(ADP-ribose) polymerase (PARP) cleavage, and flow cytometric apoptosis analysis showed that genistein at 30 μ M caused cell death via a typical apoptotic pathway in both cell lines. Western blot analysis revealed that the expression of Bax and p21^{WAF1} was up-regulated in both H460 and H322 cells treated with genistein. More importantly, significant up-regulation of p53 was detected in genistein-treated H460 cells, while no change in p53 expression was observed in H322 cells that underwent the same genistein treatment. These results suggest that genistein induces apoptosis in NSCLC cells through p53 independent pathway and, thus, may act as an anticancer agent regardless of the status of p53 in cancer cells.

Similar results have been observed in prostate cancer cells treated with EGCG. By using Western blot analysis, Gupta et al. found that EGCG treatment resulted in a dose-dependent increase of p53 in LNCaP cells (carrying wild-type p53), but not in DU145 cells (carrying mutant p53) [80]. They also found that EGCG

induced stabilization of p53, which caused an up-regulation in its transcriptional activity, thereby resulting in the activation of its downstream targets such as p21^{WAF1} and Bax and the induction of apoptosis. In a human liver cancer cell line, EGCG also significantly increased the expression of p53 and p21^{WAF1} protein, and this contributed to cell cycle arrest [81].

Several studies examined the potential effects of I3C and DIM on the proliferation and induction of apoptosis in human prostate cancer cell lines with different p53 status. They found that induction of apoptosis by I3C was p53-independent [82]. Also, induction of p21^{WAF1} expression by DIM was independent of estrogen-receptor signaling and p53 [83].

6. Effects of chemopreventive agents on AR

It has been found that androgen receptor (AR) signaling pathway plays important roles in the carcinogenesis and cancer progression through regulation of transcription of androgen-responsive genes [84] (Fig. 2). Several chemopreventive agents including genistein, I3C, DIM, and curcumin, have been found

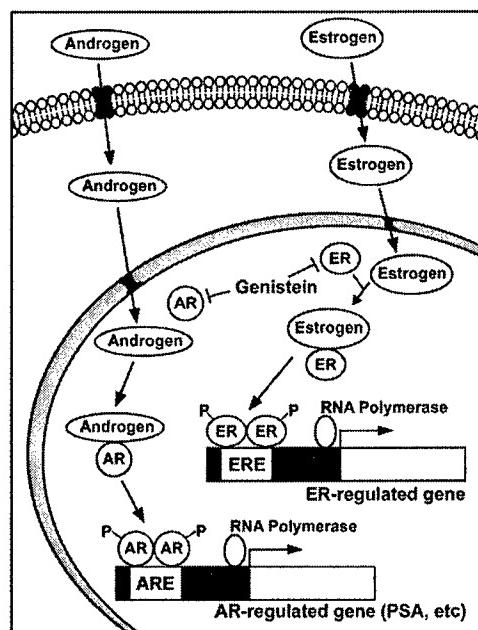


Fig. 2. AR and ER signaling pathways and the effects of genistein on the pathways.

to regulate the molecules in AR signaling pathway when they were used to inhibit growth of cancer cells.

Prostate specific antigen (PSA), one of the androgen-responsive genes, is a clinically important marker used to monitor diagnosis, progression, and prognosis of patients with prostate cancer. By using Western, Northern blots and EMSA analysis, we found that genistein at low concentration ($<10 \mu\text{M}$) transcriptionally down-regulated AR, decreased nuclear protein binding to androgen-responsive element (ARE) and, thereby, inhibited the transcription and protein expression of PSA in androgen-sensitive LNCaP cells [85,86] (Fig. 2). However, higher concentrations (10–50 μM) of genistein were needed to significantly inhibit PSA secretion in androgen-insensitive VCaP cells without alterations in the AR expression and ARE binding activity. These results suggest that genistein may be a powerful agent in the inhibition of PSA. We further transfected a PSA promoter-reporter construct into LNCaP and VCaP cells followed by treatment with or without genistein (0.5–50 μM) in the presence of media with or without R1881, a synthetic androgen. We found that genistein inhibited PSA synthesis in prostate cancer cells through both androgen-dependent and androgen-independent pathway, suggesting that genistein may act as a chemopreventive and/or therapeutic agent for prostate cancer irrespective of androgen responsiveness. Fritz et al. found that dietary genistein down-regulated expression of AR in the rat prostate at concentrations comparable to those found in humans on a soy diet [87]. Down-regulated AR expression may be responsible for the lower incidence of prostate cancer in populations on a diet containing high levels of phytoestrogens. Another chemopreventive agent EGCG also showed a dose-dependent inhibition of cell growth in both androgen-insensitive DU145 and androgen-sensitive LNCaP cells [80].

Similar to our results about the effects of genistein on AR pathway in prostate cancer cells, Le et al. also reported that DIM inhibited cell proliferation, endogenous PSA transcription, and intracellular and secreted PSA protein expression induced by dihydrotestosterone (DHT) in LNCaP cells [88]. They found that DIM inhibited androgen-induced androgen receptor (AR) translocation into the nucleus. Results of receptor binding assays indicated that DIM was a strong competitive inhibitor of DHT binding to the AR, suggesting that DIM is a strong androgen antagonist

in human prostate cancer cells. Gupta et al. also reported that apigenin treatment resulted in a significant decrease in AR protein expression along with a decrease in intracellular and secreted forms of PSA in LNCaP cells [33].

The effects of curcumin on cell growth, activation of signal transduction, and transforming activities in both androgen-dependent and -independent cell lines have been evaluated. Nakamura et al. have found that curcumin down-regulates transactivation and expression of AR and AR-related cofactors (AP-1 and NF- κ B), and reduces colony forming ability in soft agar [89]. A number of curcumin analogues was evaluated as potential androgen receptor antagonists against two human prostate cancer cell lines, PC-3 and DU-145, in the presence of androgen receptor (AR) and androgen receptor coactivator, ARA70 [90]. The results showed that some curcumin analogues possessed potent anti-androgenic activities and were superior to hydroxyflutamide, which is the currently available anti-androgen for the treatment of prostate cancer. Structure–activity relationship (SAR) studies demonstrated that some moieties seem to be important factors related to the anti-androgenic activity. These results suggest that these compounds may serve as a new class of anti-androgen agents to control androgen receptor-mediated prostate cancer growth.

7. Effects of chemopreventive agents on ER

Many environmental chemicals have been found to be estrogenic and have been shown to stimulate the growth of ER-positive human breast cancer cells [91,92]. Because it is difficult to avoid human exposure to environmental estrogens, it is important to develop dietary strategies to prevent the stimulated growth of breast tumors by environmental estrogens. Isoflavone has a close similarity in structure to estrogen, and has been known as phytoestrogen. Because of the structural similarity to estrogen, isoflavones have been believed to exert their effects through ER signaling pathway. However, experimental study has found that isoflavones at different concentration may exhibit different effects [93]. Genistein at concentrations $\leq 1 \mu\text{M}$ may induce breast cancer cell proliferation by estrogenic agonistic properties, while genistein at concentrations $\geq 5 \mu\text{M}$ may prevent hormone-dependent growth of breast can-

cer cells by potential estrogen-antagonistic activity. Fritz et al. found that dietary genistein down-regulated expression of ER- α and - β in rat at concentrations comparable to those found in humans on a soy diet [87]. Recent studies from Chen et al. [94] showed that genistein at 50 and 100 μ M significantly arrested the growth of MCF-7 cells at G2/M phase and down-regulated mRNA expression of ER α , suggesting that the inhibitory action of genistein on human breast cancer cells appears to be partially mediated by the alteration of estrogen receptor-dependent pathways. However, experimental studies also showed that isoflavones exert their inhibitory effects on ER-negative MDA-MB-231 breast cancer cells [95] and hormone-independent cancer cells [78,79,96–99]. These results suggest that isoflavones may exert their effects through ER-dependent (Fig. 2) or independent pathway.

I3C has been known to be a negative regulator of estrogen. When cells were treated with I3C and genistein, a synergistic effect of I3C and genistein was observed on the increase in GADD (growth arrest and DNA damage) expression, the induction of apoptosis, and the decrease in gene expression driven by ER α in MCF-7 breast cancer cells [100]. I3C significantly repressed the transcriptional activity of ER α , the estradiol-activated ER α signaling, and the expression of the estrogen-responsive genes, pS2 and cathepsin-D [101]. These results suggest that anti-tumor activities of I3C are associated not only with its regulation of estrogen activity and metabolism, but also its modulation of ER transcription activity. However, I3C has also been found to inhibit the expression of CDK6 and induces a G1 cell cycle arrest of human breast cancer cells, independent of estrogen-receptor signaling [102].

The effects of curcumin on ER signaling pathway have been investigated in ER-positive human breast cancer line MCF-7 and ER-negative human breast cancer line MDA-MB-231 [103]. The results showed that curcumin inhibited the proliferation of both ER-positive and ER-negative cells. The antiproliferative effect of curcumin was estrogen dependent in ER-positive MCF-7 cells. It has been reported that curcumin inhibited the expression of ER downstream genes including pS2 and TGF- α in ER-positive MCF-7 cells, and this inhibition was dependent on the presence of estrogen [17]. However, curcumin also exerted strong anti-invasive effects in vitro in ER-negative MDA-MB-231 breast cancer cells, and this effect was

not estrogen dependent [17]. These results suggest that curcumin may exert its chemopreventive effects through ER-dependent or -independent pathway.

Because both genistein and curcumin showed inhibitory effects on ER-positive and -negative breast cancer cells, the inhibitory effects of a combination of curcumin and genistein were studied in ER-positive human breast cancer cells (MCF-7 and T47D) and ER-negative MDA-MB-231 cells [103]. The results showed that combination of curcumin and genistein significantly inhibited the growth of ER-positive cells. For ER-negative MDA-MB-231 cells, the IC50 for curcumin was 17 μ M, which was reduced to 11 μ M in the presence of 25 μ M genistein. Curcumin and genistein also induced drastic changes in the morphological shape of both ER-positive and -negative cells. These results suggest that combination of natural plant compounds may have stronger preventive and therapeutic effects against the growth of breast cancers.

EGCG has been found to bind to ER α and ER β , and elicit ER-mediated gene expression in vitro. It has been found that EGCG at higher dose is anti-estrogenic for ER α , however, it is estrogenic for ER α and also for ER β at lower doses [104]. The in vitro and in vivo studies have demonstrated that polyphenolic catechins (EGCG and ECG) from green tea bind to ER α and ER β , and inhibited breast cancer cell proliferation and tumor growth, but only EGCG elicited ER-mediated gene expression [105], suggesting that polyphenolic catechins may exert their chemopreventive effects through ER-dependent or independent pathway.

8. Summary and perspectives

The data from in vivo human and animal studies and in vitro experiments clearly indicate that natural chemopreventive agents exert their inhibitory effects on carcinogenesis and tumor progression. These effects have been believed to be mediated through the regulation of cell signaling pathways including NF- κ B, Akt, MAPK, p53, AR, and ER pathways. As we discussed earlier, there are cross-talks between these pathways. Natural chemopreventive agents could exert their effects on these pathways separately or sequentially. By modulating cell signaling pathways, chemopreventive agents activate cell death signals and induce apoptosis in precancerous or cancer cells, resulting in the inhibi-

tion of cancer development or progression. It appears that the effects of these chemopreventive agents are not affected by the endogenesis molecular status such as p53 mutations and ER status of cancer cells. However, the regulation of cell signaling pathways by natural chemopreventive agents is important event in the prevention of cancers. More in depth in vitro and in vivo experiments are needed to fully elucidate the molecular mechanisms of action of chemopreventive agents in future studies.

Acknowledgments

The authors' work cited in this review was funded by grants from the National Cancer Institute, NIH (5R01CA101870-02, 1R01CA108535-01, and 5R01C A083695-04 awarded to FHS) and a grant from the Department of Defense (DOD Prostate Cancer Research Program DAMD17-03-1-0042 awarded to FHS).

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